

## ARTICLE / INVESTIGACIÓN

## Study on the anti-microbial effect of Sinigrin against some pathogenic bacterial species

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**Abstract:** The increasing anti-bacterial drug resistance is one of the biggest challenges facing doctors around the globe, so finding alternative treatments is one of the ideal options to overcome this problem. The cruciferous family is one of the wealthiest plants worldwide because it contains the most important secondary metabolites, glucosinolates, known for their anti-microbial properties. The present study aimed to evaluate the anti-bacterial effect of glucosinolates (Sinigrin) against eight bacterial isolates (*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Actinomyces*, *Proteus mirabilis* and *Streptococcus pneumoniae*). The current study investigated six concentrations of pure Sinigrin (100, 300, 500, 700, 900, and 1100 µg/ml). The sensitivity of bacterial isolates to various antibiotics was tested by VITIK 2DensiCheck equipment. The anti-bacterial activity of Sinigrin was assessed using the agar diffusion method, and the microtiter plate method measured the minimal inhibitory concentration (MIC). The highest anti-bacterial effect of Sinigrin was observed against *S. aureus*, *E. coli*, and *E. faecalis*. The anti-bacterial activity started as lower as 100 µg/ml, while a moderate effect was seen against *P. aeruginosa* and *K. pneumoniae* at a concentration lower than 700 µg/ml. On the other hand, Sinigrin was not effective against *Actinomyces*, *P. mirabilis*, and *S. pneumoniae*. It can be concluded from the present study that Sinigrin has an anti-bacterial effect on some isolates of bacteria which suggests the possibility of using Sinigrin as alternative medicine in the future.

**Key words:** Anti-bacterial activity, Agar well diffusion, Glucosinolates, Minimum inhibition concentration and antibiotic susceptibility, Sinigrin.

### Introduction

The appearance of antibiotic-resistant microbes to multiple anti-microbial agents has become a life-threatening issue to public health. Therefore, several actions must be taken to reduce this problem, for example, by controlling antibiotic usage, developing research for enhancing the genetic mechanisms of resistance, and developing new synthetic natural anti-bacterial drugs since the ultimate goal is to offer appropriate and efficient anti-microbial drugs to the patients<sup>1</sup>. Their botanical derivatives are safer in comparison with high efficacy<sup>2</sup>.

Antibiotics and other anti-microbial drugs have been used extensively in treating infectious disorders for many years to combat bacterial and fungal infections. Infectious organisms have been exposed to these substances on a large scale due to the widespread use of antibiotics, their repeat prescription in healthcare facilities, and an overuse of antibiotics in animal-rearing practices in agriculture. Because of this, some organisms have developed a resistance to them, and certain bacterial strains now significantly threaten overall health. Myrosinase-activated glucosinolates produce thiocyanates, isothiocyanates, and nitrile compounds. *In vitro* studies show that these chemicals are ideal for anti-bacterial testing in treating such infections<sup>3,4</sup>.

There are many diverse sources of natural anti-microbials, including plants, animals, bacteria, algae, and fungi.

Among them, the advantages of glucosinolates and the products derived from them for human nutrition, plant defense, and as powerful anti-bacterial agents have gained recognition. Plants produce thousands of phytochemicals, many of which have nutritive value in both medicinal and health-promoting properties<sup>5</sup>. Sinigrin (allyl-glucosinolate or 2-propenyl-glucosinolate) is natural aliphatic glucosinolate present in cruciferous or Brassicaceae plants such as broccoli, broccoli sprouts, cabbage, mustard, Brussel sprouts, cauliflower and many others. These plants have essential components of a healthy diet, and their pic flavor profile with several physiological processes<sup>6</sup>. To activate, the glucosinolates molecules require conversion by the myrosinase enzyme to bioactive thiocyanate, isothiocyanate, and nitrile derivatives; therefore, glucoraphanin and Sinigrin are converted into bioactive sulphoraphanin (SFN) and allyl isothiocyanate (AITCs) with fungicidal and bactericidal properties<sup>6</sup>. Melrose in 2019 illustrated sinigrin uses and benefits in biomedicine<sup>4</sup>. Previous studies showed Sinigrin's role in modulating the immune system<sup>7</sup>. Therefore, the present study aims to evaluate the potential anti-microbial activity of sinigrin secondary metabolite against different bacterial isolates, which may suggest the possibility of bringing it as an alternative treatment in the future.

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## Materials and methods

### Preparation of glucosinolates (sinigrin) solutions

Pure Sinigrin (extracted from the mustard plant/ Brassicaceae family) was purchased from Sigma-Aldrich. The stock solution of 3 mg/ml of Dimethyl sulfoxide (DMSO) (99 %, v/v) (Fluka) was prepared. Different concentrations of Sinigrin were prepared from stock solution (100, 300, 500, 700, 900, and 1100 µg/mL). The dilutions were prepared by using (DMSO) (99 %, v/v) (Fluka) aseptically. The Sinigrin was dissolved in a sterile container and shaken gently at room temperature<sup>8</sup>.

### Bacterial strains

Eight isolates of pathogenic bacterial species, including *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Actinomyces*, *Proteus mirabilis*, *Streptococcus pneumoniae* (blood agar was used in growing the fastidious isolated bacteria) were isolated and identified in the Microbiology Laboratory. Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq, in the level 2 biosafety lab. Basic biochemical tests and microscopic examination were used to identify the bacterial isolates. VITEK 2 fluorescence system (ID-GNB card) was used to confirm the identification of bacterial isolates<sup>9</sup>.

### Preparation of bacterial suspension and growth medium

All bacterial isolates were stored for a long time at -80 °C in a sterile cryovial containing 30 % glycerol (v/v, glycerol/overnight bacterial growth). Before starting the experiments, a loop full of bacterial isolates was inoculated onto Mueller-Hinton Agar (MHA) and incubated at 37 °C overnight. The suspension of bacterial growth was prepared by mixing the loop full of the isolated colony with sterile normal saline. The normal saline was used to adjust the number of bacteria to be  $1.5 \times 10^8$  c.f.u/ml by using a 0.5 McFarland tube<sup>10</sup>.

### Antibiotic susceptibility

VITEK2DensiCheck (bioMe'rieux) technology was employed to test the susceptibility of isolated bacteria to various antibiotics, Cefazolin, Ceftazidime, Ceftriaxone, Cefepime, Imipenem, Gentamicin, Ampicillin, Amoxicillin/Clavulanic Acid, Ampicillin/Sulbactam, Piperacillin/Tazobactam. The CLSI 2021 manual was followed for the selected drugs<sup>11</sup>.

### The anti-bacterial effect of Sinigrin against bacterial isolates

#### Diffusion method

The anti-microbial effect of different concentrations of Sinigrin was evaluated by measuring their clear inhibition zone against eight bacterial isolates cultured onto Mueller-Hinton Agar plates. A hundred microliter of standard bacterial inoculum  $1.5 \times 10^8$  of each bacterial isolate was separated onto Mueller-Hinton Agar plates. A sterile crock-poorer made several 0.45 mm diameter wells on Mueller-Hinton Agar plates. Fifty microliters of the serial concentrations (100, 300, 500, 700, 900, and 1100 µg/mL) of Sinigrin were put into the six wells, and 50 µl of DMSO was placed in the seventh well (as control). The plates were

incubated overnight at 37 °C. Scales were used to measure the clear inhibition zones.

### Minimum inhibition concentration (MIC) of Sinigrin

The minimum inhibition concentration (MIC) of pure Sinigrin was determined using a microtiter plate. A hundred microliters of each concentration of Sinigrin (100, 300, 500, 700, 900, and 1100 µg/mL) was added into the wells of microtiter plates, 100 µl of sterile tryptic soya broth (double concentration of TSB) and 10 µl of overnight growth of bacterial isolates ( $1.5 \times 10^8$  c.f.u/ml). In control wells, 100 µl of DMSO was used instead of the sinigrin solution. Three duplicates of each trial were made. The microtiter plates were shaken gently and incubated overnight at 37 °C<sup>10</sup>.

### Statistical analysis

Statistical analysis was done by using Origin 8 software. The data were expressed as means ± SE. The chi-square was used to evaluate the difference between the diameters of the test (different concentrations of Sinigrin) and the diameters of wells that were filled with DMSO (control negative). A value of  $P < 0.05$  was considered to be statistically significant.

## Results

### The anti-microbial effect of Sinigrin

The anti-bacterial effect of Sinigrin was estimated in this study against eight bacterial isolates that were isolated and diagnosed in the microbiology laboratory at the Department of Biology, College of Science, University of Baghdad. Table 1 shows that the different concentrations of Sinigrin have affected the growth of five bacterial isolates (*S. aureus*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and *E. faecalis*) but not against three isolates (*Actinomyces*, *S. pneumoniae*, and *P. mirabilis*). It was shown that the highest anti-bacterial effect of Sinigrin was seen in the case of *S. aureus* starting from the lowest concentration of 100 µg/ml though the effect was not in a concentration-dependent manner. Similarly, in the case of *E. coli* but the highest concentrations of Sinigrin (900 µg/ml and 1100 µg/ml) were non-significant. In the cases of *P. aeruginosa* and *K. pneumoniae*, the anti-bacterial effect of Sinigrin starts at 700 µg/ml concentration upwards, and the effect was in a concentration-dependent manner. In the case of *E. faecalis*, the effect of Sinigrin appeared at low concentrations, i.e., at 100 µg/ml, 300 µg/ml, and 500 µg/ml. No anti-bacterial effect of Sinigrin was observed at high concentrations, i.e., at 700 µg/ml, 900 µg/ml, and 1100 µg/ml. Finally, the growth of *Actinomyces*, *S. pneumoniae*, and *P. mirabilis* isolates was not affected (Figure 1).

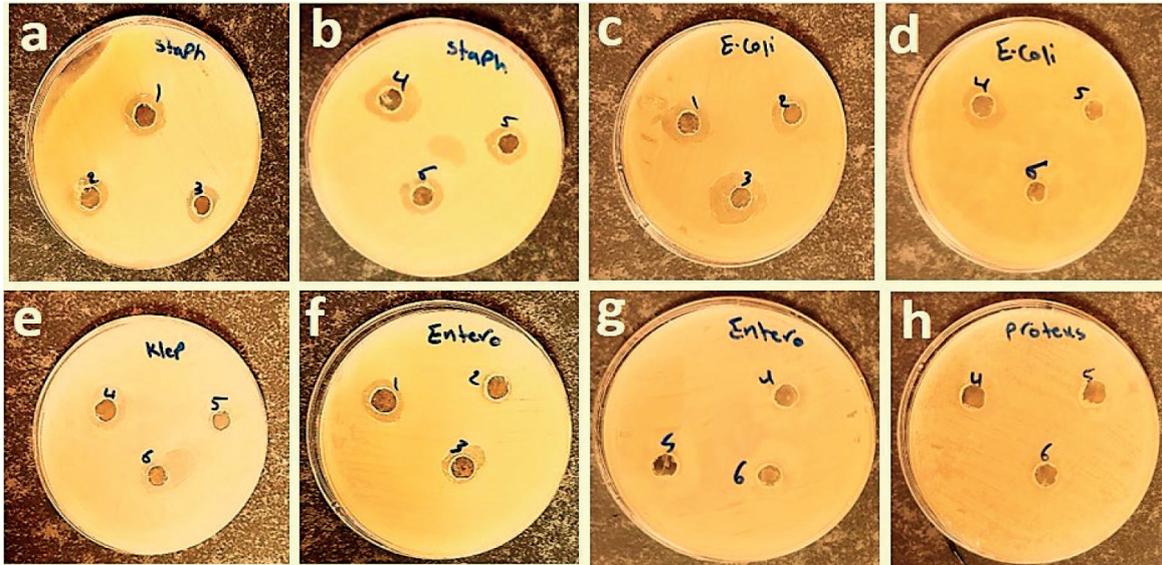
### MICs of Sinigrin against the bacterial species

The MIC method was used to evaluate the anti-microbial effect of pure sinigrin standard solution against different bacterial isolates of *S. aureus*, *E. faecalis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *Actinomyces*, *P. mirabilis*, and *S. pneumoniae*. The current study showed that the MICs of Sinigrin against *S. aureus*, *E. coli*, and *E. faecalis* were 300 µg/ml, while the MICs of Sinigrin against *K. pneumoniae* and *P. aeruginosa* were 300 and 700 µg/ml respectively. The MIC method showed no anti-bacterial effect of Sinigrin against *Actinomyces*, *P. mirabilis*, and *S. pneumoniae*.

No	Bacterial species	100 µg/ml	300 µg/ml	500 µg/ml	700 µg/ml	900 µg/ml	1100 µg/ml
1	<i>S. aureus</i>	15 ± 1.2	12 ± 1.2	12 ± 1.1	17 ± 1.3	14 ± 2.4	15 ± 1.1
2	<i>E. coli</i>	15 ± 1.4	14 ± 2.1	20 ± 2.2	14 ± 1.5	NS	NS
3	<i>P. aeruginosa</i>	NS	NS	NS	14 ± 1.9	15 ± 1.8	20 ± 1.7
4	<i>K. pneumoniae</i>	NS	NS	NS	14 ± 1.7	14 ± 1.5	18 ± 1.3
5	<i>E. faecalis</i>	11 ± 1.5	13 ± 2.4	15 ± 1.5	NS	NS	NS
6	<i>Actinomyces</i>	NS	NS	NS	NS	NS	NS
7	<i>S. pneumoniae</i>	NS	NS	NS	NS	NS	NS
8	<i>P. mirabilis</i>	NS	NS	NS	NS	NS	NS

NS: non-significant inhibition zone

**Table 1.** Anti-microbial activity of different concentrations of Sinigrin expressed as the diameter of inhibition zones in millimeters (mm) against 8 bacterial species. Each treatment was performed in triplicate.



**Figure 1.** The well-diffusion method was used to measure the anti-bacterial effect of different concentrations of Sinigrin (well 1, 100 µg/ml; well 2, 300 µg/ml; well 3, 500 µg/ml; well 4, 700 µg/ml; well 5, 900 µg/ml; well 6, 1100 µg/ml) in Mueller-Hinton Agar (MHA) that was inoculated (spared) previously inoculated with a and b (*S. aureus*); c and d (*E. coli*); e (*K. pneumoniae*); f and g (*E. faecalis*); h (*P. mirabilis*).

No	Bacterial species	MICs (µg/ml)
1	<i>S. aureus</i>	300
2	<i>E. coli</i>	300
3	<i>P. aeruginosa</i>	700
4	<i>K. pneumoniae</i>	300
5	<i>E. faecalis</i>	300
6	<i>Actinomyces</i>	Not effect
7	<i>S. pneumoniae</i>	Not effect
8	<i>P. mirabilis</i>	Not effect

**Table 2.** Minimum inhibition concentrations (MICs) of Sinigrin against 8 bacterial species. Each treatment was performed in triplicate.

## Discussion

Previous studies were focused on the treatment application of Sinigrin because of the safe and non-toxic effect of this substance as it is extracted from plant<sup>12</sup>. Several studies highlighted the therapeutic impact of Sinigrin; Tanaka *et al.* (1992) established the anticancer effect of Sinigrin<sup>13</sup>, while the anti-inflammation effect was attributed by Lee and Lee (2015)<sup>14</sup>. As for the anti-oxidant activity, it was reported by Ippoushi *et al.* (2010). The main effect of this herbal extract was the anti-microbial effect<sup>15</sup>. Several studies reported the anti-bacterial effect of Sinigrin. Other investigators highlighted the anti-bacterial impact of Sinigrin against *E. coli*<sup>16</sup>, *Bacillus subtilis*, and *Listeria monocytogenes*<sup>17</sup>, hence the development of resistance to the conventional antibiotic by pathogenic bacteria makes it necessary to find alternative anti-microbials to eradicate them<sup>18</sup>.

The use of different serial concentrations was done according to the manufacturer company of sinigrin phytochemicals are routinely classified as anti-microbials based on susceptibility tests that produce inhibitory concentrations in the range of 100 to 1,000 µg/mL, which is why this range was used in the present study<sup>19,20</sup>. In the current study, eight bacterial isolates were isolated and identified using biochemical tests and VITEK 2 DensiCheck (bioMe'rieux) technology. The diffusion method was used to check the anti-bacterial effect of different concentrations of Sinigrin. As illustrated above, the results showed that Sinigrin has an anti-bacterial effect on five isolates. Sinigrin is not usually an anti-microbial substance; when it is enzymatically hydrolyzed from allyl isothiocyanate, it exhibits potent anti-microbial activity against food spoilage and pathogenic organisms<sup>21</sup>. Allyl isothiocyanate molecules showed anti-microbial effects against *E. coli* O157:H7 at low pH. Based on

the results that proteins and sulphhydryl compounds could suppress various isothiocyanates, it was hypothesized that the mechanism of isothiocyanates' anti-microbial activity was related to the intracellular inactivation of sulphhydryl-enzymes<sup>9,22</sup>. It is known that the thioredoxin system has an essential role in DNA formation, which suggests that the anti-bacterial activity of allyl isothiocyanate could be related to the inhibition of DNA formation. Allyl isothiocyanate inhibits the catalysis of thioredoxin reductase and acetate kinase, which are responsible for essential metabolic reactions in bacteria. Thus, it can be proposed that allyl isothiocyanate has many targeted anti-microbial activities, as they can cause enzymatic inhibition and membrane damage<sup>16,17</sup>. A study by Herzallah and Holley (2015) evaluated the use of carboxymethyl cellulose (CMC) nanoparticulate on the anti-microbial activity of CMC films containing Sinigrin against *E. coli* O157:H7 on fresh beef<sup>23</sup>. An investigation by Lara-Lledo and their group found that pure Sinigrin after hydrolysis can interfere with the metabolism of bacteria and inhibit the monocytophenes growth<sup>14,18</sup>.

In the novelty of the current study, different concentrations of pure Sinigrin were used to check the anti-bacterial effect of Sinigrin against other bacterial isolates, and two methods to approve the anti-bacterial effect of Sinigrin *in vitro* were used as well<sup>13</sup>.

## Conclusions

The current study has shed light on one of the most important natural compounds, glucosinolates. Glucosinolates (from the Brassicaceae family) and their hydrolysis products (such as Sinigrin) have decisive bioactive benefits, one of which is their anti-microbial properties. We have discussed Sinigrin's anti-bacterial effect (at low concentrations) against different bacterial isolates such as *S. aureus*, *E. coli*, and *E. faecalis*. In contrast, moderate concentrations were effective against *P. aeruginosa* and *K. pneumoniae*. Further studies on glucosinolates and their hydrolysis products are recommended to investigate their anti-microbial effect, which can be a valuable approach to enhance the therapeutic activities of such critical secondary metabolites.

## Author Contributions

Alaa M. Hasan conducted the research experiments and wrote the manuscript, and Jenan A. Ghafil, conducted the research experiments, data interpretation, statistical analysis, and writing the manuscript.

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## Institutional Review Board Statement

This work was approved by the Institutional Review Board Statement of the Department of Biology, College of Science, University of Baghdad (No. 1045).

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## Conflicts of Interest

The authors declare no conflict of interest.

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