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## Evaluation of Bioactive Compounds on Different Extracts of *Linum Usitatissimum* and Its Antimicrobial Properties against Selected Oral Pathogens

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## Evaluation of Bioactive Compounds in Different Extracts of *Linum usitatissimum* and their Antibacterial Properties

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### Abstract

**Background:** Antibacterial agents from natural sources have been suggested as alternatives for treating infectious diseases due to their low side effects. Flaxseed (*Linum usitatissimum*) exhibits some antibacterial properties, but its effects against oral pathogens are poorly understood. This study investigated flaxseed extracts' antibacterial effects against such pathogens. **Methods:** Non-polar and polar extracts of flaxseed, with *n*-hexane, dichloromethane (DCM) and methanol (MeOH), were prepared by sequential Soxhlet extraction. All extracts were qualitatively screened through gas chromatography–mass spectrometry to detect bioactive compounds. Antibacterial activities of flaxseed extracts with different volumes per disc (Oxoid, Badhoevedorp, Netherlands) (5, 7, 10, 12 and 15 µL/disc) were evaluated against four different oral pathogens: *Streptococcus pyogenes*, *Streptococcus mutans*, *Lactobacillus casei* and *Enterococcus faecalis*, using the disc diffusion method. Flaxseed extracts' inhibitory activities against the tested oral pathogens were examined by measuring the inhibition zone diameter. **Results:** Polar extract (methanolic extract) demonstrated significant antibacterial activity ( $p < 0.01$ ) against all tested oral pathogens, with inhibition zones comparable to those for penicillin. In contrast, *n*-hexane and DCM extracts demonstrated variable antibacterial activities. **Conclusions:** This study suggests that *Linum usitatissimum* methanolic extract exhibited the best inhibitory activity on all tested strains compared to the other extracts.

**Keywords:** antibacterial agents, methanolic extract, *Linum usitatissimum*

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### Introduction

Despite being largely preventable, oral diseases remain a major public health issue worldwide. A global review of oral health, published by the World Health Organization (WHO), indicated oral health is still a global problem, even though some countries have achieved great improvements.<sup>1</sup> In fact, dental caries and periodontal disease are the most common infectious diseases afflicting humans and are linked to various kinds of Gram-negative and Gram-positive bacteria. The wide-spread and rapid emergence of multiple drug-resistant bacteria has prompted scientists across the world to develop novel antimicrobial agents. Healthcare systems in the 21st century face a major challenge in dealing with the resistance of these bacteria. Although traditional healers have used plants to cure and prevent infectious diseases since ancient times, modern scientists are now attempting to duplicate their successes through clinical-based research, including in the pursuit of medications against multiple drug-resistant bacteria. Studies on traditional medicinal products have revealed

the critical roles played by plants that are rich in various metabolites such as alkaloids, flavonoids, tannins and terpenoids, for which empirical evidence of their antimicrobial properties has been reported.<sup>2,3</sup> In this study, we focused on assessing flaxseed extracts' antimicrobial activities on four selected oral pathogens: *S. pyogenes*, *S. mutans*, *L. casei* and *E. faecalis*.

Flaxseed or linseed with the scientific name *L. usitatissimum*, is a valuable herb belonging to the Linaceae family.<sup>4</sup> The terms 'flaxseed' and 'linseed' are commonly used to refer to flax, but have slightly different meanings. The former refers to flax when consumed as food by humans, whereas the latter is associated with flax used for animals feeding and industrial purposes.<sup>5,6</sup> Flaxseed is believed to have originated in Egypt, and it has been cultivated worldwide for its oil and fibre for many years.<sup>7</sup>

In recent years, many researchers have paid special attention to flaxseed since studies have shown its

pronounced benefits on health, including via anticancer and antibacterial effects.<sup>8</sup> Studies also showed that flax lignans, flaxseed oil and fibres help treat many diseases such as atherosclerosis, cardiovascular disease, arthritis, cancer, diabetes, osteoporosis and autoimmune and neurological disorders.<sup>6</sup> Previous studies revealed that its nutritious components include fibre,  $\alpha$ -linolenic acid (ALA, omega-3 fatty acid), lignans, proteins as well as fixed oil.<sup>6,9</sup> A previous study also revealed that flaxseeds consist of 35%–45% oil, 20%–25% protein and a small portion of cyanogenic glycosides, with linoleic and linolenic acids being the main components of the oil.<sup>10</sup> The aim of this study is to investigate flaxseed extracts' antibacterial effects against oral pathogens.

## Methods

**Plant material and extract preparation.** *Linum usitatissimum* seeds were collected from the University of Philadelphia in Jordan. Dried flaxseeds (100 g) were ground, using a blender, and extracted using a Soxhlet extractor with solvents of increasing polarity, beginning with *n*-hexane, followed by dichloromethane and finally methanol. The powdered plant samples were extracted successively with *n*-hexane, dichloromethane, and methanol. Each extraction was carried out continuously for 8–10 hours.<sup>11</sup> The extracts produced with different organic solvents were evaporated using a rotary evaporator to remove the remaining organic solvents, leaving a small yield of extracted plant material in the glass-bottomed flask. Then, the extract was further dried in a fume hood until a constant mass was obtained.

**Fatty acids into fatty acid methyl esters (FAME).** FAME analysis by gas chromatography–mass spectrometry (GC-MS) – Here, 14% methanolic boron trifluoride (BF<sub>3</sub>/MeOH) was used to derivatize fatty acids into fatty acid methyl esters (FAME). FAME preparation was performed in line with MPOB Test Methods p3.4: 2004.<sup>12</sup> GC-MS analyses of *L. usitatissimum* FAME were performed using a GC Clarus™ SQ 8 Perkin Elmer system. The gas chromatograph was connected to a mass spectrometer (GC-MS) under the following conditions: Column Elite-5MS fused silica capillary column (30 mm length × 0.25 mm inner diameter × 0.25 μm film thickness, composed of 100% dimethylpolysiloxane), operating in electron impact mode (ionizing energy) at 70 eV. Helium (99.9%) was used as carrier gas at a constant flow rate of 1 mL/min and an injection volume of 2 μL was employed (split ratio of 10:1). The injector temperature was 250 °C and the ion-source temperature was 280 °C. The oven temperature was programmed to start at 70 °C (isothermal for 6 min) with an increase of 6 °C/min to 280 °C. Mass spectra were taken at 70 eV, with a scan interval of 0.5 s and fragments from 45 to 450 Da. The total GC running time was 60 min.

**Bacterial strains.** The microorganisms used in this study included *S. mutans* (ATCC 25175), *S. pyogenes* (ATCC 19615), *L. casei* (ATCC 4646), and *Enterococcus faecalis* (ATCC 29212) as oral pathogens. All bacterial strains were incubated overnight in Mueller Hinton Broth (MHB) (Oxoid) under aerobic conditions at 37 °C to be used as an inoculum. The suspensions' turbidity was adjusted to  $1.5\text{--}3 \times 10^8$  cells/ml, which corresponds to an absorbance of 0.08–0.10 at a wavelength of 625 nm.<sup>13</sup>

**Agar disc diffusion.** The antibacterial tests were performed using the disc diffusion method. Agar plates were prepared using sterile Mueller Hinton agar (Oxoid). The bacterial inoculum was evenly spread onto the surface of the agar plates using a sterile cotton swab. Crude extracts of *L. usitatissimum* (with *n*-hexane, dichloromethane and methanol) were pipetted at different volumes (5, 7, 10, 12 and 15 μL) onto sterile blank discs of 6 mm in diameter (Oxoid, Badhoevedorp, Netherlands), which were allowed to dry before being impregnated onto agar plates inoculated with bacteria. Since the unit of volume per disc was used for evaluation, an appropriate blank disc must be able to take the maximum amount of tested volume. Standard antibiotics, namely, ampicillin and penicillin at 10 mg/mL, were used as positive controls, while broth was used as a negative control for all tested bacteria. Diffusion of the extracts and antibiotics was allowed at room temperature, using a blower in a biosafety cabinet until full drying had occurred. The agar plates were then incubated at 37 °C for 6–18 h. The presence of a clear zone (inhibition zone) around the discs on the plates was observed, and the average diameter of these zones was measured to assess antibacterial activity. The presence of an inhibition zone denoted that inhibitory activities had taken place. All tests were performed in triplicate and the results are presented as mean ± SE.

## Results

*L. usitatissimum*'s potential to be used to cure infectious diseases prompted us to specifically study its antibacterial activity against oral pathogens. The four oral pathogens selected for use in this study were *S. mutans*, *E. faecalis*, *S. pyogenes* and *L. casei*. The extracts' chemical compositions were investigated using gas chromatography–mass spectrometry (GC-MS) analyses in order to understand the nature of the bioactive principles and their mechanisms of action. The in vitro antibacterial activity of *L. usitatissimum* were quantitatively measured by determining the inhibition zones' diameter. The inhibition zones produced by the test organisms indicated their susceptibility to the flaxseed extracts. The inhibitory results of antibacterial activity are shown in Table 1.

The elution order for *n*-hexane analysis was as follows: palmitic acid, linoleic acid, linolenic acid and stearic acid. The elution order for methanolic extract analysis: myristic acid, palmitic acid, linoleic acid, linolenic acid and stearic acid. The results of the quantitative determination of the levels of these compounds are shown in Tables 2–4.

GC-MS analysis of the *n*-hexane extract showed the presence of five main compounds, as presented in Table 2. The fatty acid present at the highest rate was linolenic acid (76.79%), followed by linoleic acid (12.79%), palmitic acid (6.3%) and stearic acid (2.1%). This is in accordance with a previous study.<sup>14</sup> On the other hand, GC-MS analysis of dichloromethane extract led to identifying various classes of compound. Specifically, the nine most prevalent components were hexadecanoic acid (4.04%), oleic acid (11.46%), oleic acid, 3-hydroxypropyl ester (5.19%), 9-octadecanoic acid (Z)-,2,3-dihydroxypropyl ester (12.22%), 4-hexyl-1-(7-methoxycarbonylheptyl) bicyclo[4,4,0] deca-2,5,7-triene (17.21%), trimethylsilyl derivative of 2-monoolein (11.16%), 3,4-diphenyl- 6-methyl- 7,8-(1,4-dimethoxybenzo)-9-oxatricyclo [4.2.1.0(2,5)] non-3-ene (3.33%), 9,19-cyclolanost-24-en-3-ol, acetate, (3 $\beta$ ) (34.67%) and 9,19-cycloergost-24(28)-en-3-ol,4,14-dimethyl-acetate(3 $\beta$ ,4 $\alpha$ , 5 $\alpha$ ) (0.71%) (Table 3). Besides that, five phytochemicals were characterized and identified in methanolic extract. The bioactive compounds, with their retention time (RT) and percentage of relative composition, are presented in Table 4. Specifically, Table 4 shows five phytochemical

compounds identified in methanolic extract; linolenic acid was the most abundant at 53.01%, followed by palmitic acid (17.10%), linoleic acid (16.44%), stearic acid (3.74%), and finally myristic acid (0.71%). For the antibacterial assessment, the results regarding the inhibitory zones for *S. mutans*, *E. faecalis*, *S. pyogenes* and *L. casei* are presented in Table 1. The studies were performed in triplicate, and the results are expressed as mean  $\pm$  SE. From this study, *n*-hexane and dichloromethane extracts demonstrated poor *in vitro* antimicrobial profiles against *S. mutans*, *E. faecalis*, *S. pyogenes* and *L. casei*. On the other hand, methanolic extract showed potent activity against all four of these species. None of the strains demonstrated resistance to this extract, and the inhibitory zone significantly increased in a dose dependent manner (Figure 1). The inhibition zone diameter for all tested bacteria treated with methanolic extract differed significantly at  $p < 0.01$ . However, the test extract showed better antimicrobial activity against *S. pyogenes* at all concentrations, followed by those against *L. casei*, *E. faecalis*, and finally *S. mutans*. Finally, *n*-hexane and dichloromethane extract showed no inhibition zone for all bacteria, except for *S. pyogenes* with a weak inhibition zone. For *n*-hexane extract, the inhibition zone was  $6.7 \pm 0.67$  mm (mean  $\pm$  SE) for concentrations of 5 and 7  $\mu$ L/disc and  $7.0 \pm 1$  mm for concentrations of 10, 12 and 15  $\mu$ L/disc, while for dichloromethane extract, the values were  $6.7 \pm 0.67$  mm for concentrations of 5 and 7  $\mu$ L/disc,  $7 \pm 1$  mm for a concentration of 10  $\mu$ L/disc,  $8 \pm 1$  mm for a concentration of 12  $\mu$ L/disc and  $9 \pm 1$  mm for a concentration of 15  $\mu$ L/disc.

**Table 1.** Antibacterial activities of *L. usitatissimum* against oral microorganisms controlled with zones of inhibition in millimeters using disc diffusion method (mean  $\pm$  SE)

Variable	<i>p</i>	Plant extract ( $\mu$ L/disc)					Negative control	Positive control
		5	7	10	12	15		
<b><i>S. mutans</i></b>								
<i>n</i> -Hexane	-	NI	NI	NI	NI	NI		
Dichloromethane	-	NI	NI	NI	NI	NI		
Methanol	**	$9.67 \pm 0.33$	$13.67 \pm 0.88$	$15.33 \pm 0.33$	$17.33 \pm 0.67$	$19.33 \pm 0.67$	NI	$29.67 \pm 0.33$
<b><i>E. faecalis</i></b>								
<i>n</i> -Hexane	-	NI	NI	NI	NI	NI		
Dichloromethane	-	NI	NI	NI	NI	NI		
Methanol	**	$10.33 \pm 1.33$	$12.00 \pm 1.53$	$14.33 \pm 0.67$	$16.33 \pm 0.88$	$20.67 \pm 1.45$	NI	$29.00 \pm 0.58$
<b><i>S. pyogenes</i></b>								
<i>n</i> -Hexane	-	$6.7 \pm 0.67$	$6.7 \pm 0.67$	$7.0 \pm 1$	$7.0 \pm 1$	$7.0 \pm 1$		
Dichloromethane	-	$6.7 \pm 0.67$	$6.7 \pm 0.67$	$7.0 \pm 1$	$8.0 \pm 1$	$9.0 \pm 1$		
Methanol	**	$20.00 \pm 1$	$22.30 \pm 1.45$	$23.00 \pm 1.15$	$25.00 \pm 1.15$	$27.30 \pm 1.45$	NI	$30.00 \pm 0.58$
<b><i>L. casei</i></b>								
<i>n</i> -Hexane	-	NI	NI	NI	NI	NI		
Dichloromethane	-	NI	NI	NI	NI	NI		
Methanol	**	$10.67 \pm 0.33$	$14.00 \pm 0.00$	$16.33 \pm 0.88$	$20.00 \pm 0.00$	$22.33 \pm 1.45$	NI	$26.00 \pm 0.58$

\*\* : Significant; - : Not significant; NI: no inhibition ( $6.00 \pm 0.00$ );  $p < 0.01$

**Table 2.** Phytochemical compounds identified in *n*-hexane extract of *L. usitatissimum*

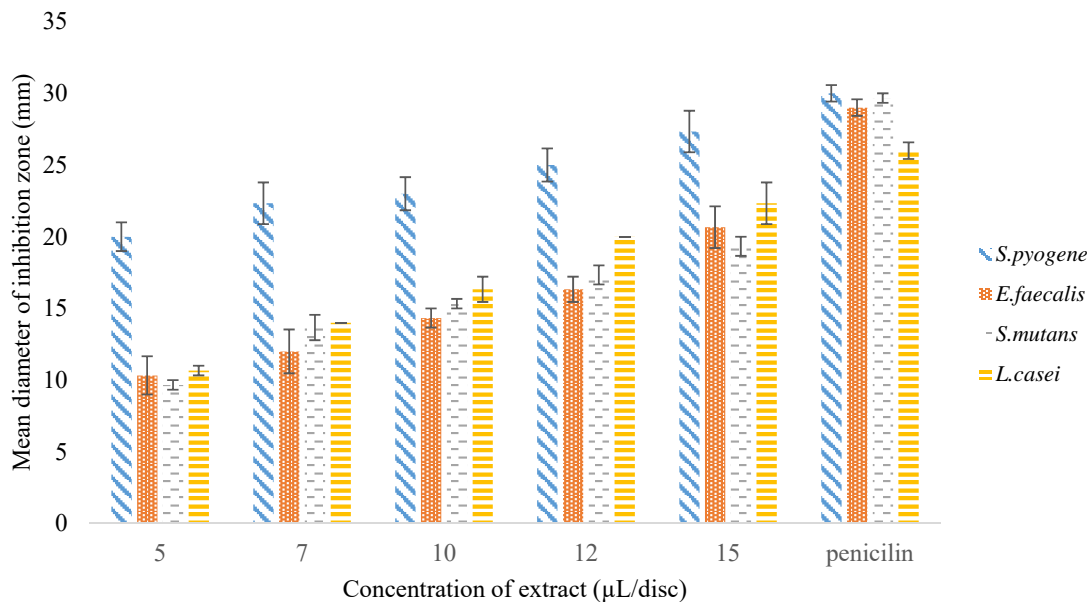
Retention time	Name of the compound	Class of fatty acid	Molecular formula	Exact mass (g/mol)	Peak area (%)	Biological activity
38.92	Palmitic acid	Saturated fatty acid	C <sub>17</sub> H <sub>34</sub> O <sub>2</sub>	270.25588	6.3	Antibacterial <sup>15</sup>
42.82	Linoleic acid	Unsaturated fatty acid	C <sub>19</sub> H <sub>34</sub> O <sub>2</sub>	294.25588	12.79	Antibacterial <sup>16</sup>
42.97	Linolenic acid	Unsaturated fatty acid	C <sub>19</sub> H <sub>32</sub> O <sub>2</sub>	292.24023	76.79	Antimalaria <sup>17</sup> and antibacterial <sup>18</sup>
43.60	Stearic acid	Saturated fatty acid	C <sub>19</sub> H <sub>38</sub> O <sub>2</sub>	298.28718	2.1	Antibacterial and antifungal <sup>19</sup>

**Table 3.** Phytochemical compounds identified in dichloromethane extract of *L. usitatissimum*

Retention time	Name of the compound	Class of compound	Molecular formula	Exact mass (g/mol)	Peak area (%)	Biological activity
35.49	Hexadecanoic acid	Saturated fatty acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	256.24023	4.04	Antimicrobial, anti-inflammatory, antioxidant and hepatoprotective <sup>20, 21</sup>
39.39	Oleic acid	Unsaturated fatty acid	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>	282.25588	11.46	Antibacterial and antifungal <sup>19</sup>
44.95	Oleic acid, 3-hydroxypropyl ester	Fatty acid	C <sub>21</sub> H <sub>40</sub> O <sub>3</sub>	340.297745	5.19	Antimicrobial and antioxidant <sup>22</sup>
45.60	9-Octadecanoic acid (Z)-,2,3 Dihydroxypropyl ester	Fatty acid	C <sub>21</sub> H <sub>40</sub> O <sub>4</sub>	356.29266	12.22	Antimicrobial, anti-inflammatory, antioxidant and hepatoprotective <sup>20, 21</sup>
49.19	4-Hexyl-1-(7-methoxycarbonylheptyl) bicyclo [4,4,0] deca-2,5,7-triene	Carboxylic acid derivative	C <sub>25</sub> H <sub>40</sub> O <sub>2</sub>	372.30283	17.21	Not identified
49.52	Trimethylsilyl derivative of 2-monoolein	Lipid	C <sub>27</sub> H <sub>56</sub> O <sub>4</sub> Si	500.371714	11.16	Antioxidant and anti-atherosclerotic <sup>23</sup>
53.87	3,4-diphenyl-6-methyl-7,8 -(1,4 dimethoxybenzo)-9-oxatricyclo [4.2.1.0(2,5)] non-3-ene	Ether derivative	C <sub>27</sub> H <sub>24</sub> O <sub>3</sub>	396.172544	3.33	Not identified

**Table 4:** Phytochemical compounds identified in methanolic extract of *L. usitatissimum* (GC-MS)

Retention time	Name of the compound	Class of fatty acid	Molecular formula	Exact mass (g/mol)	Peak area (%)	Biological activity
33.79	Myristic acid	Unsaturated fatty acid	C <sub>15</sub> H <sub>30</sub> O <sub>2</sub>	242.22458	0.71	Antibacterial <sup>24</sup>
38.92	Palmitic acid	Saturated fatty acid	C <sub>17</sub> H <sub>34</sub> O <sub>2</sub>	270.25588	17.10	Antibacterial <sup>15</sup>
42.80	Linoleic acid	Unsaturated fatty acid	C <sub>19</sub> H <sub>34</sub> O <sub>2</sub>	294.25588	16.44	Antibacterial <sup>16</sup>
42.92	Linolenic acid	Unsaturated fatty acid	C <sub>19</sub> H <sub>32</sub> O <sub>2</sub>	292.24023	53.01	Antimalarial <sup>17,18</sup>
43.60	Stearic acid	Saturated fatty acid	C <sub>19</sub> H <sub>38</sub> O <sub>2</sub>	298.28718	3.74	Antibacterial and antifungal <sup>19</sup>



**Figure 1.** Antibacterial activities of *L. usitatissimum* methanolic extract on *S. pyogenes*, *E. faecalis*, *S. mutans* and *L. casei*. Data are presented as means  $\pm$  SE

## Discussion

Phytochemicals are biologically active and naturally occurring chemicals produced by plants. Studies of flaxseed composition have been conducted widely, and all of its identified bioactive compounds exhibit various therapeutic potentials, including as antimicrobial agents. Flaxseed has nutritional and health benefits since it is rich in omega-3-fatty acids, which are extremely important for human health, as well as phenolic compounds, for which many health benefits have been suggested.<sup>25</sup> Overall, the present study revealed the presence of several types of fatty acid in the flaxseed extracts. Their concentrations, measured in *n*-hexane and methanolic extracts, are generally consistent with those reported in previous studies. One previous study reported  $\alpha$ -linolenic acid (39.9%–60.42%), linoleic acid (12.25%–17.44%), palmitic acid (4.9%–8%), oleic acid (13.44%–19.39%), and stearic acid (2.24%–4.59%).<sup>5</sup> However, slight concentration differences might be due to several factors. For example, the fatty acid composition of flaxseed oil could be influenced by environmental factors, such as planting area, climate, storage and transportation, as well as the extraction time, solvents used, and sample preparation.<sup>25</sup> Moreover, differences might also emerge due to the methods for measuring flaxseed oil components.<sup>26</sup> The significance results of all tested bacteria treated with methanolic extract could indicate that the methanolic extract of *L. usitatissimum* had broad-spectrum antimicrobial effects against oral pathogenic bacteria, which could be the basis for the folkloric use of the plant. These results are comparable

to those in a previous study which reported that the methanolic extract of flaxseed exhibited excellent antiviral activities.<sup>11</sup> Another previous study also reported flaxseed's methanolic extract was able to inhibit the growth of the Gram-negative bacterium *P. aeruginosa*.<sup>27</sup>

However, for *n*-hexane and dichloromethane extracts, the results do not parallel those of the GC-MS analysis of *n*-hexane extract, which demonstrated fatty acids (palmitic, stearic, 9-octadecenoic, linoleic and linolenic) that are suggested to have antibacterial activity<sup>7,28,29</sup> by acting as anionic surfactants and possessing antibacterial and antifungal properties at low pH.<sup>30,31</sup> However, the poor *in vitro* antibacterial profile of *n*-hexane extract is supported by a previous study that revealed the *n*-hexane extract of flaxseed was ineffective against *P. aeruginosa*.<sup>27</sup> In addition, a previous study also revealed that *n*-hexane extract did not achieve any reduction in the growth of *Escherichia coli*, *Bacillus cereus*, *Candida albicans* or *Klebsiella pneumoniae*.<sup>32</sup>

## Conclusions

In conclusion, as oral infection remains a burden to healthcare systems worldwide, alternatives for preventing biofilm-induced caries and periodontitis in order to improve quality of life are crucial. The present work emphasizes the role of a natural product, flaxseed, in inhibiting bacterial infections. GC-MS screening demonstrated the presence of various bioactive compounds in flaxseed that possess antibacterial properties. The study also showed that *L. usitatissimum*'s methanolic

extract demonstrated better antibacterial activities against *S mutans*, *S pyogenes*, *E faecalis*, and *L casei*, compared to *n*-hexane and dichloromethane extracts. However, advanced studies are required to fully validate the antimicrobial efficacy of *L. usitatissimum* extract.

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## Conflict of Interest Statement

The authors declare no conflicts of interest associated with the work described in this manuscript.

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