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Effects of fatty acids in super critical fluid extracted fixed oil from Withania somnifera seeds on Gram-negative Salmonella enterica biofilms

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Highlights

- Supercritical CO_2 extracted W. somnifera seed oil (WSSO) is rich in fatty acids
- WSSO destabilizes cell membrane and outer membrane of Gram-negative S. enterica
- WSSO prevents bacterial motility
- WSSO inhibits biofilm formation and maturation and disrupts mature biofilm

Abstract

Background

: Biofilms are responsible for the growing resistance of bacteria to antibiotics, preventing the antibiotic accessibility to bacterial cells. As an alternative to antibiotics, fatty acids (FAs) have been explored for their antibiofilm activities due to their diffusible nature and ability to modulate the membrane fluidity of the bacteria. Fatty acids have been shown as anti-biofilm agents mostly for Gram-positive pathogens. Therefore, we chose to explore the anti-biofilm activity of FAs from Withania somnifera (L.) Dunal (Solanaceae) seeds fixed oil (WSSO) on the Gram-negative pathogen, Salmonella enterica. Biofilm formation in Salmonella spp enhances its resistance to antibiotics and host immune response, with consequent increase in its virulence and chronicity of infection. FAs from WSSO was reported for their effectivity against Psoriasis-like skin inflammation. Biofilms are implicated in Psoriasis pathology and anti-biofilm therapy can provide new treatment options. In view of its biological importance, we have explored the activity of WSSO against planktonic and biofilm forms of S. enterica.

Purpose

: The purpose of the current study is to evaluate the potentials of fixed fatty acids from an important medicinal plant (W. somnifera) as antibiofilm agents against a Gram-negative bacterium (S. enterica).

Methods

: Antibacterial activity of WSSO against planktonic form of S. enterica was evaluated through broth microdilution method. Antibiofilm activity in terms of prevention of biofilm initiation, inhibition of biofilm formation and disruption of mature biofilm were quantified through crystal-violet staining. WSSO induced loss of membrane integrity, and concomitant effect on S. enterica motility were assessed through quantification of intracellular potassium and nucleotide effluxes, and motility assays, respectively.

Results

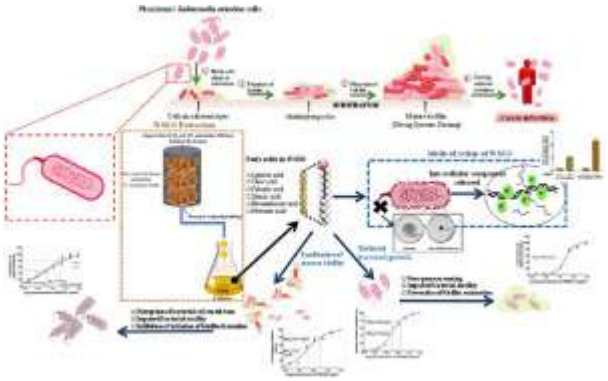
: The minimal inhibitory concentration of WSSO required for 50 % reduction in the planktonic bacterial load (IC_{50}) was 5.78 mg/ml. Cells lost their motility when treated with WSSO at IC_{50}. At a similar concentration of 6.28 mg/ml, WSSO disrupted mature biofilm of S. enterica. Bacterial cell membrane was compromised after treatment with 5.40 mg/ml WSSO as evident from potassium (K^+) ion and nucleotide effluxes.

Conclusion

: WSSO not only prevented initiation of biofilm formation and but also efficiently disrupted mature biofilm of S. enterica. Taken together, these results indicated that WSSO has the potentials to be used as an alternative antibacterial agent against Gram-negative pathogenic S. enterica. The mode of action of WSSO seems to be linked to its ability to modulate membrane

integrity and to restrain bacterial motility, most likely by its abundant and trace fatty acid contents.

Graphical abstracts



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Keywords

WSSO; Salmonella enterica; antibacterial; fatty acids; potassium efflux; nucleotide efflux; biofilm; bacterial motility

Introduction

Discovery of penicillin in 1928, closely followed by that of sulfonamide in 1935, marked the beginning of a new era in clinical microbiology. However, the emergence of penicillinase producing Staphylococcus aureus almost soon after the first clinical application of penicillin in 1940s marked the inception. As an alternative, the use of plant materials is currently recognized as an effective approach for handling this issue, one that was to make the medical system struggle despite its advancements some 90 years down the line (Saga and Yamaguchi, 2009). Antibiotics have evolved significantly over the next six decades from 1930s-1990s, and so did the bacterial resistance to them. During this time, aminoglycosides, chloramphenicol, tetracycline, macrolides, vancomycin, methicillin, nalidixic acid, first, second and third generation cephems, carbapenem, monobactams and quinolones were developed. Indiscriminate use of these drugs led to the emergence of penicillin interfering Streptococcus pneumonia (PISP), penicillin resistant S. pneumonia (PRSP), penicillinase producing, β-lactamase negative, ampicillin resistant (BLNAR) Haemophilus influenza, extended spectrum β-lactamase (ESBL) producing Gram-negative bacilli, vancomycin resistant enterococci (VREs) emerged, resistant gonococci, multidrug resistant Pseudonomas aeruginosa (MDRP) and quinolone resistant Escherichia coli. Only during the past two decades this unselective use of antibiotics has been reduced (Saga and Yamag

However, drug resistance developed by different bacterial strains till now, prevents effective treatments.

Drug resistance is conferred due to changes in bacterial genome that enhances their fitness in the face of selection pressure from the evolving antibiotics. Changes in bacterial genomes facilitate quorum sensing and biofilm formation through diffusible signal factors (DSFs). Biofilms constitutes physical barriers made of extra-cellular polymeric substance (EPS) containing polysaccharides, proteins, extracellular DNA and lipids (Kumar et al., 2020). Bacterial cells embedded within the biofilm become inaccessible to the antibiotics, thus, making them highly resistant to antibiotic treatment. This quite often results in recurrent infections (D'Abrosca et al., 2013). As an alternative, the use of plant materials is currently recognized as an effective approach for handling this issue.

Several studies proved long chain fatty acids (LCFAs) to be bactericidal for Gram-positive bacteria and that they affect the integrity of the bacterial cell wall (Galbraith and Miller, 1973a; Sheu et al., 1975). The significant difference in the sensitivities of Gram-positive and Gram-negative bacteria towards LCFAs was believed to be due to the structure of the cell wall of the latter that prevented LCFAs from reaching the lipid bilayer of the cell membrane (Galbraith and Miller, 1973b; Greenway and Dyke, 1979; Sheu et al., 1975). This was experimentally proved in case of Salmonella typhimurium, where the outer lipid bilayer was significantly resistant to hydrophobic substances while, the protoplasts devoid of this layer were sensitive to them (Nikaido, 1976). Most of the earlier reports emphasize that effectivity of FAs as antibacterial against Gram negative bacteria is either non-existent or meagre. All these studies have been conducted with single FAs(Yoon et al., 2018).

Withania somnifera (L.) Dunal (Solanaceae), a popular medicinal herb, is extensively used in traditional Indian medicine. It is a rich source of phytocompounds like steroids (withanolides) and alkaloids many of which has been identified and extracted (Balkrishna et al., 2020; Mwitari et al., 2013; Vyas et al., 2011). Roots, leaves, and fruits of W. somnifera exhibited potent antibacterial activities against Salmonella enterica and other pathogenic bacteria which are attributed to the withanolides present in them (Alam et al., 2012; Owais et al., 2005). In an earlier study from our group, W. somnifera seeds fixed oil (WSSO) showed to be effective against psoriasis. In the light of a previous study demonstrating that innate immune system of human skin has antimicrobial lipids as an indispensable component, this observation led us to think that WSSO could be a potential antibacterial agent (Yoon et al., 2018). WSSO was obtained using CO_2 as supercritical solvent, which offers the advantages of possessing low viscosity and high diffusivity into the seeds, thus ensuring improved extractions of FAs present in trace amount. Linoleic, oleic, palmitic and stearic acids were identified as the major long chain fatty acids (LCFAs) in WSSO while ecosatrienoic and nervonic acids, which are also LCFAs, were present in trace amounts (Figure 1) (Balkrishna et al., 2020). Thus, WSSO, being a mixture of abundant and trace FAs, is anticipated to be effective against Gram negative bacteria. S. enterica infection and antibiotic

resistance are results of biofilm formation, which in turn is dependent on bacterial motility and quorum sensing. Biofilm forms through several stages. Therefore, the present study was designed to evaluate the effect of WSSO at the following stages of S. enterica biofilm development: (1) initial stages of biofilm formation (when bacterial cells attach to the substratum), (2) at the maturation stage (when the agglomerated bacterial cells secrete extracellular polymeric substance or EPS to fortify the biofilm) and (3) on mature biofilm (from which bacterial cells disperse to recolonize new surfaces). Besides, we have explored the possible mode of action of WSSO as an antibacterial and antibiofilm agent.

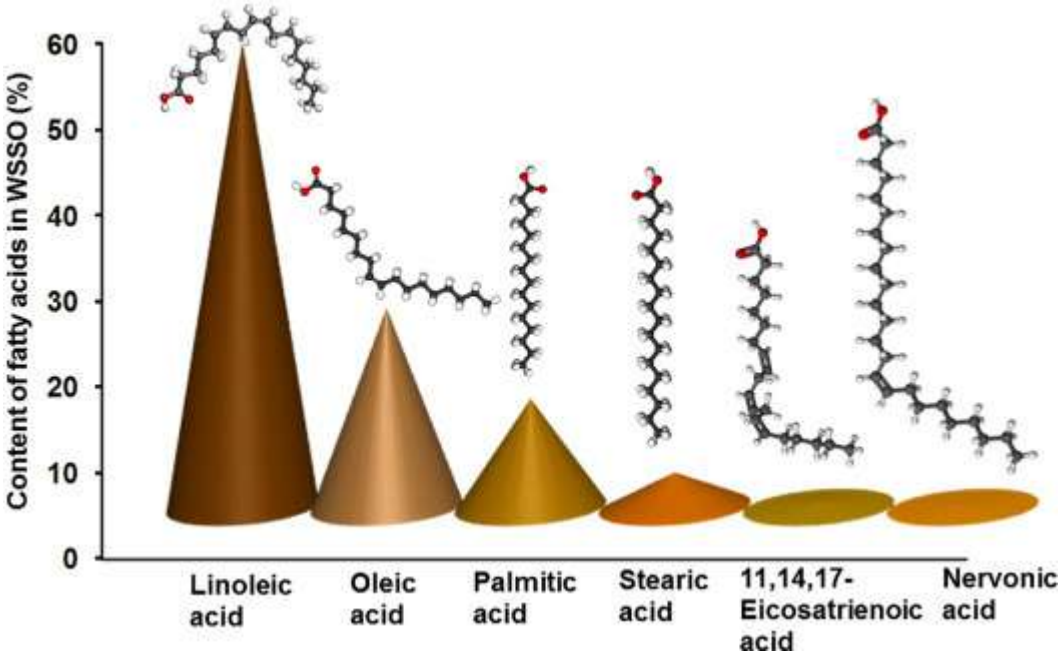

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Figure 1. Chemical composition of WSSO on GC-FID quantification.

A schematic showing the composition of WSSO in terms of percentages of different fatty acids present along with their chemical structures [Adapted from Fig 2 and Table 1 of (Balkrishna et al., 2020)].

Material and methods

Chemicals, bacteriological media, and bacterial strain

Chemicals used in this study were from Sigma-Aldrich (St Louis, MO, USA) unless otherwise mentioned. Bacteriological media were procured from Difco (BD Biosciences, San Jose, CA, USA). The pathogenic strain of S. enterica (MTCC1165) was obtained from Microbial Type Culture Collection (MTCC), CSIR-Institute of Microbial Technology (Chandigarh, India).

will refer this strain as S. enterica. Bacterial cells were reactivated from glycerol stock, first by streaking on nutrient agar plate for the single colony and subsequently, followed by overnight growth in nutrient broth at 37°C.

Antibacterial assay

Determination of inhibitory concentration through microbroth dilution method

The antibacterial potency of WSSO against S. enterica was evaluated through microbroth dilution method as per the standard guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2015). WSSO was emulsified in an equal volume of 5% DMSO containing 0.1% v/v polysorbate-80. Stock solution (180 mg/ml) was further two-fold serially diluted with 5% DMSO along with 0.1%v/v polysorbate-80 which resulted in test dilutions ranging from 0.70-90 mg/ml with reference to WSSO and 0.01–1.25 % wrt to DMSO. After dispersing 125 µl of each test dilution of WSSO in triplicate in the wells of a 96-well plate, an equal volume of bacterial suspension of S. enterica prepared in 2x Mueller Hinton Broth (MHB), containing ~ 10^5 CFU/ml was added to each well so that the final volume per well reached 250 µl. A control set having bacterial cells incubated in amount of DMSO corresponding to each dilution (0.01–1.25 %) was included to ensure that the observed inhibitory effects were not due to DMSO. The maximum concentration of DMSO to which the cells got exposed was 2.5%. The plates were incubated for 18 h at 37°C with shaking at 195 xg . Post-incubation, absorbance of these cultures was measured at 600 nm using a microplate reader (Envision, Perkin Elmer, Waltham, MA, USA). Chloramphenicol (Lupin, Mumbai, India) was used as a positive control. A negative control containing 5% DMSO along with 0.1%v/v polysorbate-80 was used. Percent bacterial growth inhibition by WSSO was calculated using the following equation:

$$\% \text{ Bacterial Growth Inhibition} = [(A_c - A_t)/A_c]\ 100$$

Where A_c: Absorbance of the control; A_t: Absorbance of test extract

Percent growth inhibition was represented graphically as a function of increasing WSSO concentrations. Using the inbuilt option of GraphPad Prism software 7.05 (San Diego, CA, USA), IC_{50} value which inhibited 50% percent of the organism tested was determined (Van Dijck et al., 2018).

Determination of bactericidal potency of WSSO

Different concentrations of WSSO 0.70-90 mg/ml were inoculated with a fixed concentration of S. enterica bacterial cells (10^5 CFU/ml) in sterile Mueller Hinton broth and incubated at 37°C for 24h. Subsequently, 50 µl of each incubated mix was spread on sterile Mueller Hinton Agar plates (without WSSO). Colony-forming units (CFU) were counted after 24h incubation at 37°C and plotted as CFU/plate versus concentration of WSSO. The lowest concentration of WSSO which

prevented bacterial growth was identified as the minimum bactericidal concentration (MBC) (Bertelloni et al., 2020). Chloramphenicol (Lupin) was used as a positive control.

Mechanism of action

Potassium (K^+) cation leakage

The K^+ leakage from S. enterica was estimated in the presence of WSSO. K^+ leakage was quantified by using a spectrophotometric method (Rajawat et al., 2014). Bacterial cells from an overnight culture were centrifuged at 8600xg for 10 min, washed twice with 1x PBS (pH 7.2) and resuspended in 1x PBS at a density of 1×10^8 cells/ml 1 ml aliquots of the cell suspension were mixed with 0.90, 3.60, 5.40 and 10.8 mg/ml and incubated at 37˚C for 0, 2, 4, 6, and 8 h. At each point, the supernatant was collected by centrifugation and mixed with 25% sodium cobaltinitrite. The sodium cobaltinitrite precipitate formed was kept at room temperature for 45 min and subsequently washed with 95% ethanol. After adding concentrated HCl to the precipitates, samples were incubated at room temperature for 20 min for deep green color formation that was quantified at 623 nm by using a UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). Chloramphenicol (30 µg/ml) (Lupin) was used as a positive control. The intensity of the green coloration was proportional to the amount of K^+ ions present in the supernatant and quantified using a standard curve.

Nucleotide efflux

Cells from logarithmic phase culture of S. enterica were washed and resuspended as 1 ml aliquots in 1x PBS buffer (pH 7.2) at a density of 1×10^8 cells/ml, followed by incubation with different concentrations (0.90, 3.60, 5.40 and 10.8 mg/ml) of WSSO for 12 h. Every 3 h, an aliquot was filtered and absorbance of the filtrate measured at 260 nm using a UV-VIS spectrophotometer (Shimadzu) (Da Silva et al., 2014). Chloramphenicol (30 µg/ml) (Lupin) was used as positive control.

Antibiofilm activity

Determination of biofilm forming capacity of S. enterica (MTCC1165)

S. enterica biofilms were grown in 96-well plates, containing 200 µl of cell suspension in MHB containing 1×10^6 cells/ml. MHB without any bacterial inoculum was included as negative control. Plates were incubated for 24 and 48 h at 37°C. Subsequently, biofilm biomass was stained with crystal violet, the stain extracted with acetic acid and absorbance measured at 570 nm. Critical OD value (ODc) was defined according to Christensen et al. (1985) and Gomes et al. (2019) as three standard deviations above the mean OD of the negative control and the biofilm production was scored as: [strong biofilm producers {(+++), OD > 4 x ODc}, moderate biofilm

producers {(++), 2 x ODc < OD < 4 x ODc}, weak biofilm producers {(+), ODc < OD ≤ 2 x ODc} and non-biofilm producers {(-), OD ≤ ODc}] (Christensen et al., 1985; Gomes et al., 2019).

Determination of inhibitory effect of WSSO on biofilm formation

S. enterica cell suspension aliquots of 125 µl in MHB containing 1×10^6 cells/ml were mixed with different concentrations of WSSO (0.70, 1.40, 2.80, 5.62, 11.25, 22.5, 45 and 90 mg/ml) and incubated for 24 h at 37°C. Biofilm biomass was determined through crystal violet staining as described above. Percent inhibition of biofilm formation was calculated with respect to the untreated control.

Determination of preventive effect of WSSO on biofilm maturation

Cells were first allowed to incubate for 24 h at 37°C without shaking following which they were mixed with different concentrations of WSSO (0.70, 1.40, 2.80, 5.62, 11.25, 22.5, 45 and 90 mg/ml) and incubated for another 24 h at 37°C. Biofilm biomass was determined through crystal violet staining as described above and percent preventive efficiency of biofilm maturation was calculated with respect to the untreated negative control.

Determination of disruptive effect of WSSO on mature biofilm

In this case, the cells at same density as mentioned earlier were allowed to form mature biofilm by incubating them at 37°C for 48 h without shaking. The mature biofilms were then treated with different concentrations of WSSO (0.70, 1.40, 2.80, 5.62, 11.25, 22.5, 45 and 90 mg/ml) for another 24 h at 37°C. The biofilm biomass degradation was assayed by the crystal violet staining method (Famuyide et al., 2019).

Staining of biofilm

Crystal violet staining

After following the above incubation periods, biofilms were stained according to a previous report (Gomes et al., 2019). All planktonic cells were carefully removed, and adhered/biofilm cells were washed twice with NaCl 0.9% (w/v), and then fixed for 15 min at 37°C by adding 250 µl of methanol, and subsequently, cells were air-dried after discarding the excess methanol. 250 µl of 1% (v/v) crystal violet (CV) was added to each well for 5 min. The stained adherent/biofilm cells were air-dried for 2 h, following which, 250 µl of 33% (v/v) acetic acid was added to extract the dried CV stain. Absorbances of the resulting violet solutions were measured at 570 nm. Un-inoculated wells stained with CV were used as blank controls. Percent inhibition of biofilm formation and maturation and percent disruption of mature biofilm were determined using the following equation

$$\%\ \text{bacterial biofilm inhibition} = [(A_c - A_t)/A_c]\ 100$$

where A_c: Absorbance of the untreated sample; A_t: Absorbance of treated sample

Data represented as percent inhibition of bacterial biofilm formation (BIC or biofilm inhibition concentration) or percent disruption of the mature biofilm (BEC or biofilm eradication concentration) as a function of increasing concentrations of WSSO (Van Dijck et al., 2018). 50% inhibitory (BIC_{50} or BEC_{50}) concentrations for each assay was determined using an in-built option provided in GraphPad Prism 7.0.

Gram staining of biofilm

Gram staining of microbial biofilms was performed as reported earlier (Haney et al., 2018). For staining of biofilms, control cells and test cells were grown on coverslips placed in 35 mm petri plates. 125 µl of bacterial suspension in MBH containing 1×10^6 cells/ml, was added on the coverslips and incubated at 37°C for 48 h without any shaking, following which 0.70, 1.40, 2.80, 5.62, 11.25, 22.5, 45 and 90 mg/ml of WSSO was added and incubated further at 37°C for 24 h without agitation. The coverslips were Gram-stained, to verify the effect of WSSO on the viable cells embedded within the biofilm. Stained coverslips were mounted on clean glass slides by applying 40% glycerol and imaged using a bright field microscope (AxioScope A1, Carl Zeiss MicroImaging GmbH, Oberkochen, Germany) under 100x objective.

Bacterial motility assay

Motility inhibition of S. enterica in the presence of WSSO was determined through swimming and swarming assays. Lauria Bertani (LB) plates enriched with 0.5% (w/v) glucose, and containing 0.20% and 0.50% bacteriological agar, respectively, were used for swimming and swarming assays (Chelvam et al., 2014). 5 µl of an overnight culture of S. enterica with 5.89 mg/ml and without WSSO were placed on the agar surface of the specific plates. Both types of plates were kept for incubation at 37°C for 24 h. Uninhibited motility of the bacteria was visible as expanded circular bacterial surface around the initial spot of application of the bacterial culture. The radius of this expanding circle, measured as the distance traveled by the bacteria, depicted the extent of bacterial motility. The plates were imaged and distances measured from three independent experiments for each type of motility were represented as mean ± SE.

Statistical analysis

Statistical significance of the observed differences between the average potassium ion and nucleotide effluxes of various groups was analyzed through one-way ANOVA followed by Dunnett's post-hoc test and indicated with ** for $p < 0.01$ when compared to control. Paired t-test was used to evaluate the statistical significance of the observations for motility assays and indicated with * and ** for $p< 0.05$ and $p< 0.01$, respectively. Two-way ANOVA with Bonferroni's post-hoc test was used to assess the statistical significance of the observations regarding inhibitory effect of WSSO on biofilm formation and were represented with ** and *** for $p < 0.01$ and $p < 0.001$, respectively. All statistical analyses were done using GraphPad Pris

Results

Determination of IC_{50} and IC_{80}

Concentrations of Chloramphenicol and WSSO against S. enterica MTCC1165 that inhibited 50 and 80 % of bacterial growth was determined through microbroth dilution method. Chloramphenicol at 14.6 µg/ml (IC_{50}) and 48.5 µg/ml (IC_{80}) (Figure 2A) while WSSO at concentration 5.78 mg/ml (IC_{50}) and 12.41 mg/ml (IC_{80}) (Figure 2B) inhibited 50 and 80% of the bacterial growth, respectively. Bactericidal potency of WSSO was evaluated as the efficiency with which colony forming units (CFU) were formed after WSSO treatment. The CFU was counted and plotted against different concentrations of the positive control, chloramphenicol concentrations (Figure 3A) or WSSO (Figure 3C). We observed that even at 90 mg/ml, a few CFUs were still visible, suggesting a high MBC value for WSSO (Figure 3C) which reflected as ~80 % inhibition in the bacterial growth at 100 mg/ml of WSSO (Figure 3D). Bactericidal activity of chloramphenicol was observed at higher concentration (Figure 3A & B).

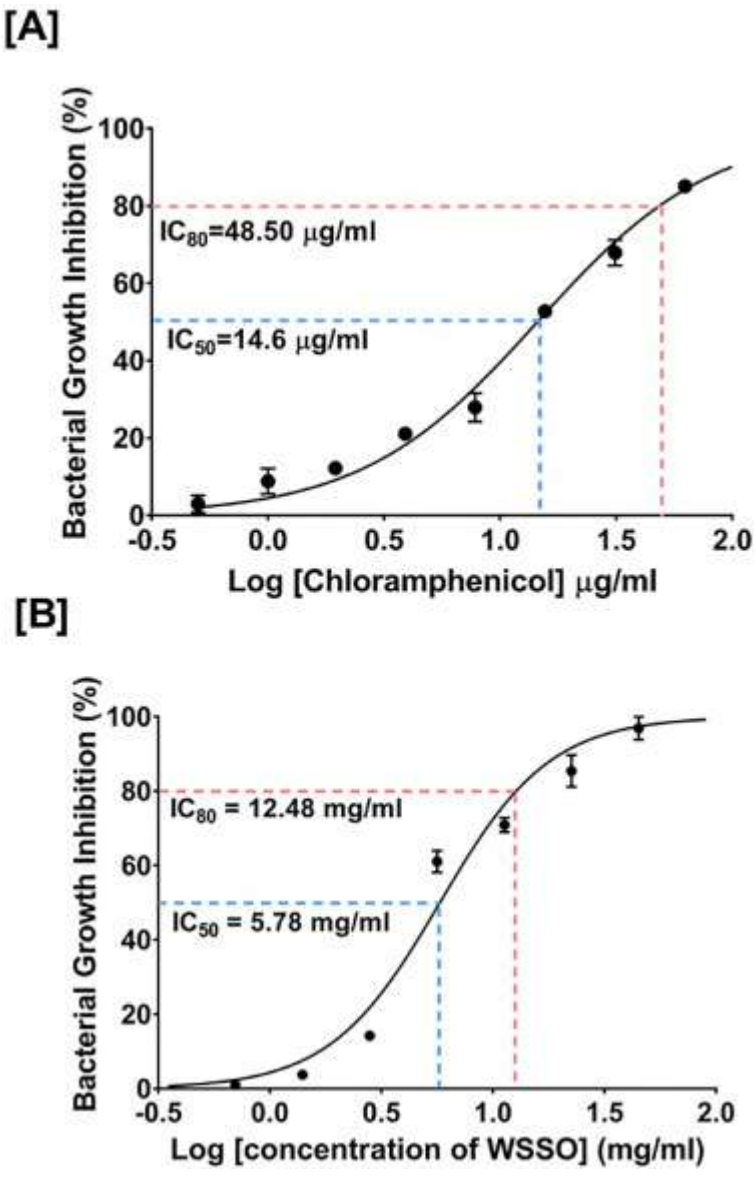

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Figure 2. Antibacterial effect of WSSO on S. enterica

[A, B] Dose-response curves showing a normalized dose-dependent inhibitory effect of positive control chloramphenicol (A) and WSSO (B) on the growth of S. enterica cells as evaluated through microbroth dilution method. Concentrations responsible for 50 (IC_{50}) and 80 (IC_{80}) % growth inhibitions, as determined through non-linear regression analysis, are mentioned.

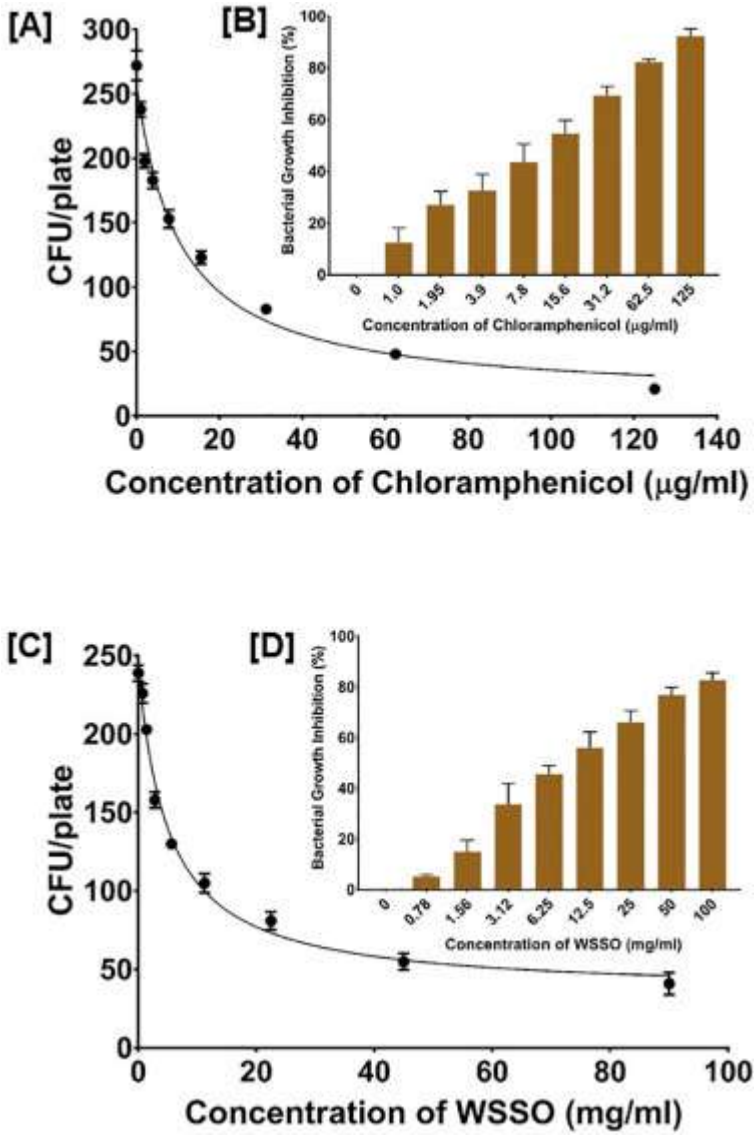

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Figure 3. Bactericidal potency of WSSO against S. enterica

[A, C] Rectangular hyperbolic plots of CFU/plate versus different concentrations of positive control chloramphenicol and WSSO. [B, D] Percent growth inhibition versus concentration bar graphs showing the effects of increasing concentrations of chloramphenicol (B) and WSSO (D) on S. enterica growth.

Intracellular potassium (K^+) ion leakage

The mechanism behind the antibacterial effect of WSSO on S. enterica was evaluated by investigating the effect of WSSO treatment on the bacterial cell membrane. Leakage of intracellular ions is indicative of damage in the cell membrane which can be detected through the presence of K^+ ions in the medium. So, growth media of cells treated with different concentrations of WSSO were tested for the presence of K^+ ions. Untreated cells were taken as negative control while cells treated with chloramphenicol (that is known to disrupt the bacterial membrane), were included as a positive control. . An indirect quantification method involving the colorimetric measuring of acid precipitates of potassium cobaltinitrite was employed. The amount of the precipitated K^+ ions were determined in parts per million (ppm) using a standard curve. We did not detect any K^+ ion in the medium of untreated cells throughout the experiment even after 8 h. Lower concentrations of WSSO 0.9 and 3.6 mg/ml also did not show any K^+ ions leakage in the media. However, K^+ ions were detectable 3 h onwards in the medium from cells treated with near IC_{50} concentration 5.40 mg/ml of WSSO and the measured levels were significantly higher when compared to untreated cells. K^+ ion efflux in chloramphenicol treated cells was nearly 4 times higher than the untreated within 2 h of treatment, kept on increasing till 8 h, and became almost 6 times that of untreated. K^+ ion efflux in cells treated with 10.8 mg/ml (double to the determined IC_{50} concentration) followed a trajectory almost overlapping that of the positive control (Figure 4A). The fold change in K^+ ions after 8 h in positive control calculated concerning untreated showed 5.7 times more efflux of these cells. Cells treated with different concentrations of WSSO exhibited dose-dependent fold increase in K^+ ion efflux; treatment with 10.8 mg/ml of WSSO showing comparable (4.8) fold change to that of positive control (Figure 4B).

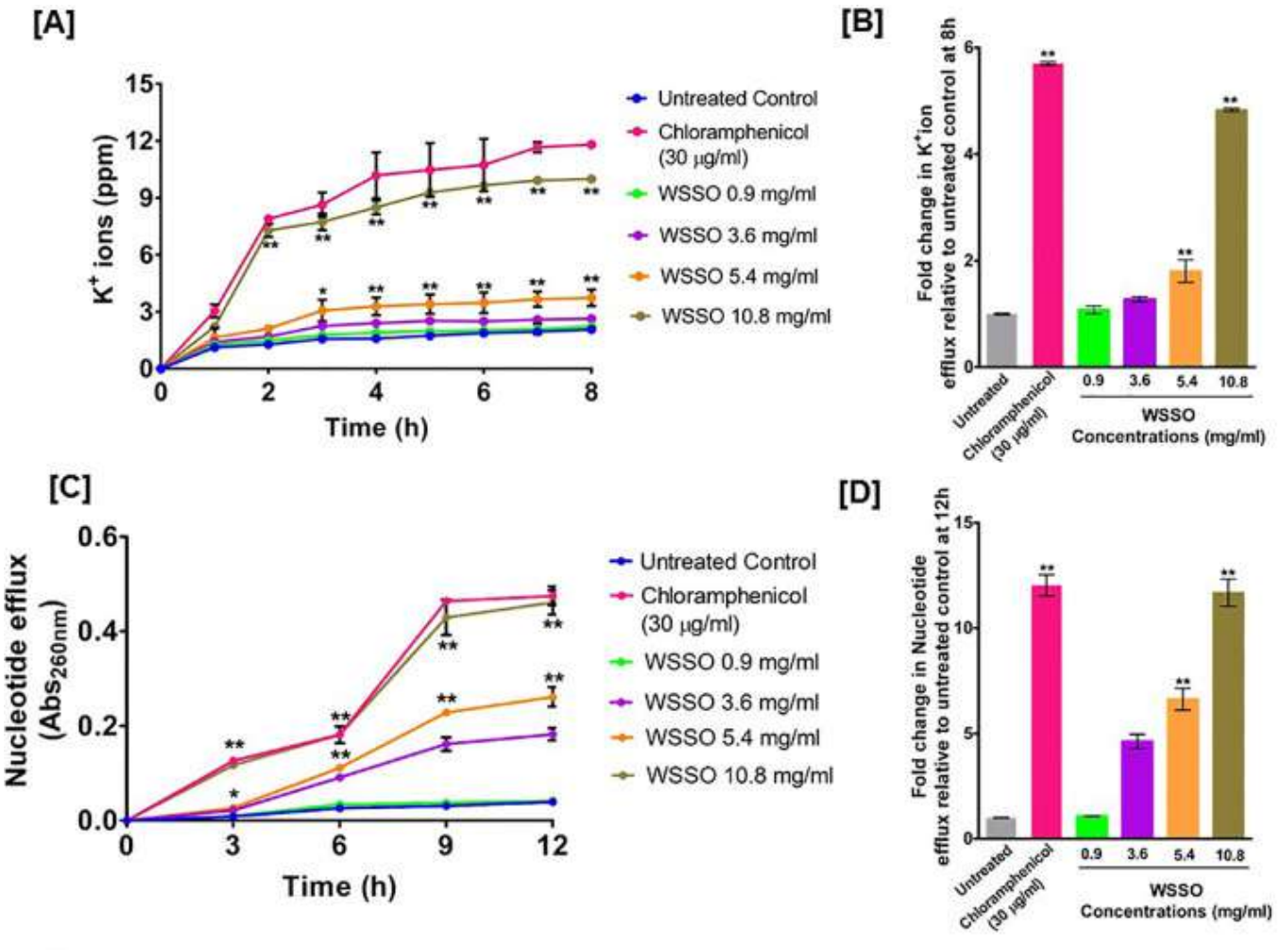

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Figure 4. WSSO treatment promotes efflux of intracellular ions and nucleotides from the bacterial cells

[A, C] Comparative line graphs showing concentration and time dependent effect of WSSO on intracellular K^+ (A) and nucleotide (C) leakage/efflux. [B, D] The observed fold change in intracellular K^+ (B) and nucleotide (D) leakage/efflux after 8 and 12 h, respectively, of treatment with different concentrations of WSSO. Data represented as mean ± SE. Statistical significance determined through one-way ANOVA with Dunnett's multiple comparison test where * and ** represent $p < 0.01$ and 0.001, respectively, when compared to untreated control in case of (A & C) and chloramphenicol in case of (B & D).

Nucleotide efflux

Along with the K+ leakage quantification, nucleotide efflux is also one of the most common methods for monitoring the damages to cell membranes. Therefore, if WSSO damages the cell membrane in S. enterica, the presence of nucleotides will be evident in the mediu

media of cells treated with different concentrations of WSSO were tested for the efflux of nucleotides. Untreated cells were taken as negative control while cells treated with chloramphenicol were included as a positive control. Lower concentration of WSSO (0.9 mg/ml) did not show any nucleotide efflux in the medium. However, nucleotide efflux was detectable 6 h onwards in the medium from cells treated with near IC_{50} concentrations (3.60 and 5.40 mg/ml) of WSSO and the measured levels were significantly higher when compared to untreated cells. Nucleotide efflux in chloramphenicol treated cells was nearly 7 times higher than the untreated within 6 h of treatment, kept on increasing till 12 h, and became almost 12 times that of untreated. Nucleotide efflux in cells treated with 10.8 mg/ml (double the determined IC_{50} concentration) followed a trajectory almost overlapping that of the positive control (Figure 4C). The fold change in nucleotide efflux after 12 h in positive control calculated concerning untreated showed 12 times more efflux of these cells. Cells treated with different concentrations of WSSO exhibited dose-dependent fold increase in nucleotide efflux; treatment with 10.8 mg/ml of WSSO showing fold change (11.7) comparable to the positive control (Figure 4D).

Inhibitory effect of WSSO on the motility of S. enterica

Effect of WSSO on the motility of S. enterica was measured for their surface swimming (media with 0.2% agar) and swarming (media with 0.5% agar) ability. A 24 h incubation resulted in distinct circular bacterial zones in both swimming and swarming plates with untreated cells indicating unhindered motility of bacteria. However, following treatment with 5.78 mg/ml WSSO, no bacterial zones were observed showing that the motility of the cells has been jeopardized (Figure 5A). By measuring the radius of the bacterial zones, we determined the extent of motility inhibition WSSO could inflict. We found at 50% microbial inhibitory concentration (IC_{50}) of 5.78 mg/ml, WSSO reduced swimming and swarming almost equally (~2.7 and ~ 3.0 folds, respectively) (Figure 5B).

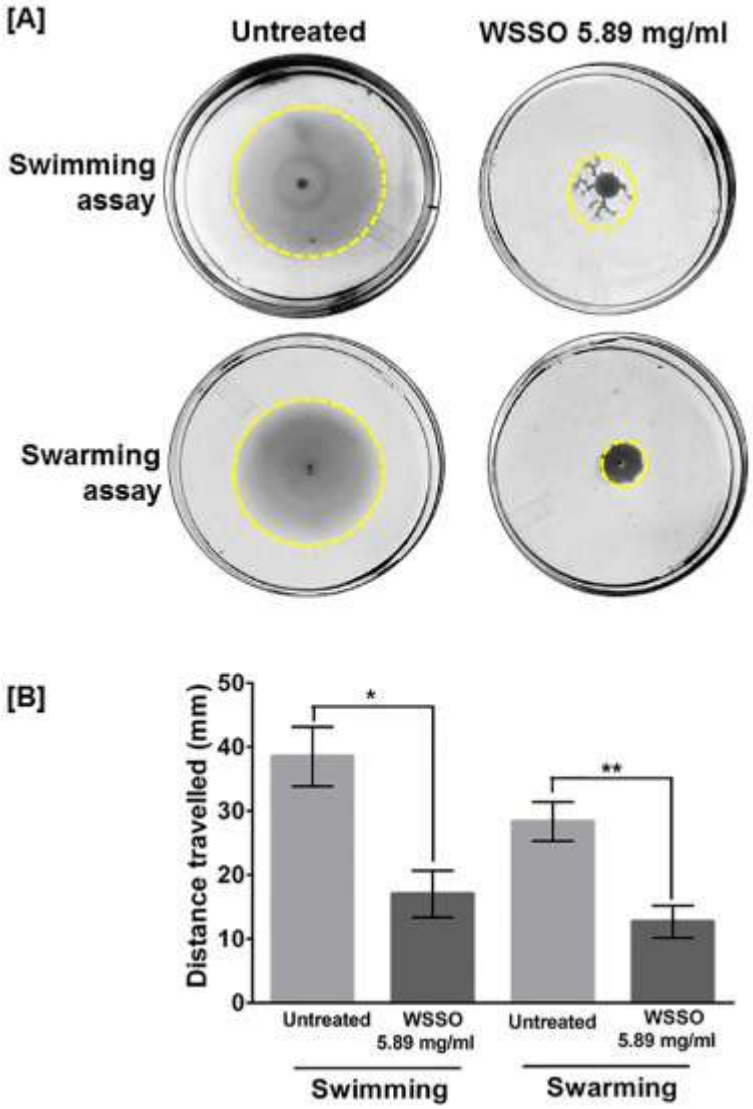

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Figure 5. Detrimental effect of WSSO treatment on bacterial motility

[A] Representative digital images (inverted) of swimming and swarming assay plates showing the effect of WSSO (at 5.89 mg/ml) on S. enterica motility. [B] The observed attenuation of bacterial motility in response to WSSO treatment is represented as distance travelled by the bacteria as determined by measuring the radius of the circular bacterial zone formed by treated and untreated cells. Data represents the mean ± SE from three independent experiments. Statistical significance determined through paired t-test where * and ** represent $p < 0.05$ and 0.01, respectively, when compared to untreated to respective controls.

Antibiofilm activity

For the determination of the antibiofilm activity of WSSO, it was necessary to test the biofilm formation tendency of S. enterica strain used. We observed that S. enterica MTCC1165 can form moderate to strong biofilms depending on the duration of incubation. With an incubation of 24 h, the biomass of the formed biofilm as determined according to Gomes et al., 2019, who qualifies it to be a moderate one, while, an incubation of 48 h resulted in a strong biofilm (Gomes et al., 2019) (Table 1).

Table 1. S. enterica (MTCC1165) biofilm formation after 24 and 48 h cultivation

Time	Control Mean[a] (OD±SD)	Biofilm Mean[b] (OD±SD)	Ratio (b/a)	Biofilm formation[#]
24h	0.104±0.045	0.364±0.050	3.5	++
48h	0.101±0.021	0.420±0.067	4.15	+++

OD, optical density; SD, Standard deviation;

[#] Gradation of biofilm formation as moderate biofilm producer (++) and strong biofilm producer(+++)are as per Gomes et al., 2019 (Gomes et al., 2019)

We first checked whether WSSO treatment is capable of inhibiting biofilm formation, for which, we allowed the bacterial cells to adhere to the bottom of the wells before incubating them with different concentrations of the oil for 24 and 48 h without shaking. At designated time points, the biofilm biomasses were CV stained and quantified. We observed that WSSO prevented biofilm formation in a dose-dependent manner. Apparently, longer incubation did not have any visible advantage; rather, it seemed to be unnecessary as evident from statistically significant increased inhibitory effects, for concentrations 2.80, 22.5 and 45 mg/ml. However, the concentrations near IC_{50} did not show difference for 24 and 48 h incubations (Figure 6A). We plotted a normalized dose-response curve to determine the corresponding BIC_{50} values for 24 and 48 h incubations. Although the values were in the same range, the one for 24 h (5.56 mg/ml) was lesser than that for 48 h (6.87 mg/ml), implying that a 24 h incubation might be sufficient to prevent the biofilm formation. In order to prevent biofilm formation with an efficiency of 80 % by 24 h, one might need to incubate with 19.24 mg/ml (BIC_{80}) of WSSO (Figure 6B). Next, we checked whether WSSO can prevent biofilm maturation. From our experiments to determine the biofilm forming capacity of the S. enterica strain under study, we figured out that following a 24 h incubation, the cells formed moderate biofilm which after another 24 h becomes strong. This indicated that by 24 h, the biofilm reaches the maturation phase. Therefore, our experimental design allowed the cells to form biofilm for 24 h before treatment with WSSO for another 24 h. WSSO co

biofilm maturation in a dose-dependent manner. However, its efficiency of preventing biofilm maturation was less compared to its efficiency in inhibiting the initiation of biofilm formation. This was evident from a higher BIC_{50} value of 15.66 mg/ml for prevention of biofilm maturation (compared to 5.56 mg/ml for 24 h or 6.87 mg/ml for 48 h incubation for inhibition of biofilm initiation study) (Figure 6C). We further evaluated whether WSSO could disrupt and eradicate mature biofilms. For this strong mature biofilm formed through 48 h incubation of bacterial cells was treated with WSSO for 24 h. A treatment with 6.29 mg/ml of WSSO calculated from the normalized dose-response curve was enough to eradicate 50% of the mature biofilm (BEC_{50}) (Figure 7A). Similarly, for an 80% biofilm eradication (BEC_{80}), 15.57 mg/ml of the oil. WSSO treated and untreated mature biofilms were Gram stained and imaged and our observations from these images could be corroborated with our quantitative data mentioned above (Figure 7B).

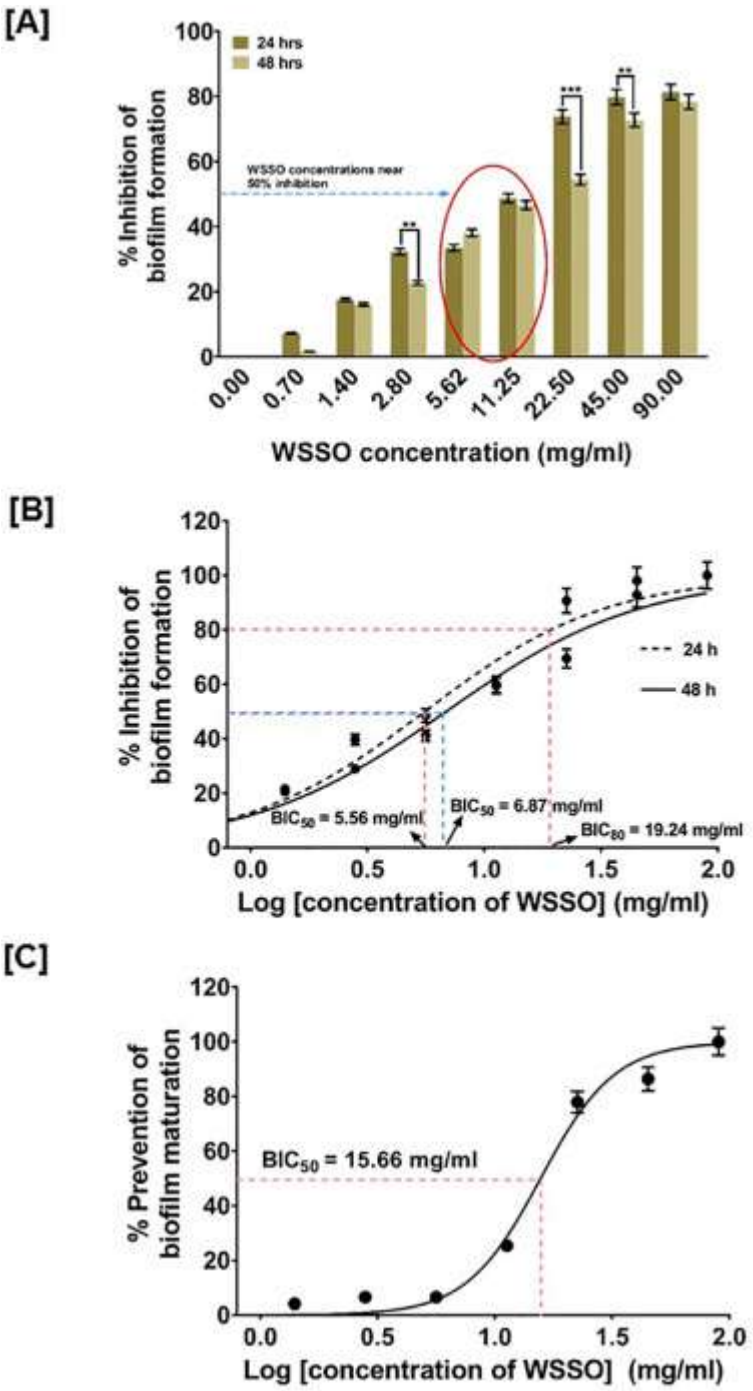

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Figure 6. WSSO inhibits initiation of biofilm formation more efficiently than preventing its maturation

[A] Comparative graphical representation of dose-dependent responses of WSSO on inhibition of initiation of biofilm formation over 24 and 48 h incubation. Doses near 50 % inhibition of bacterial growth are encircled in red open circle. ** and *** respectively denote $p < 0.01$ and 0.001 from two-way ANOVA with Bonferroni's post-hoc test. [B] Normalized dose-response curve of the above showing the doses sufficient for 50 and 80 % inhibitions of the inhibition of biofilm formation after 24 (broken line) and 48 h (solid line) incubations. [C] Normalized

curve exhibiting the preventive effect of WSSO on biofilm maturation with the dose required for 50 % effect.

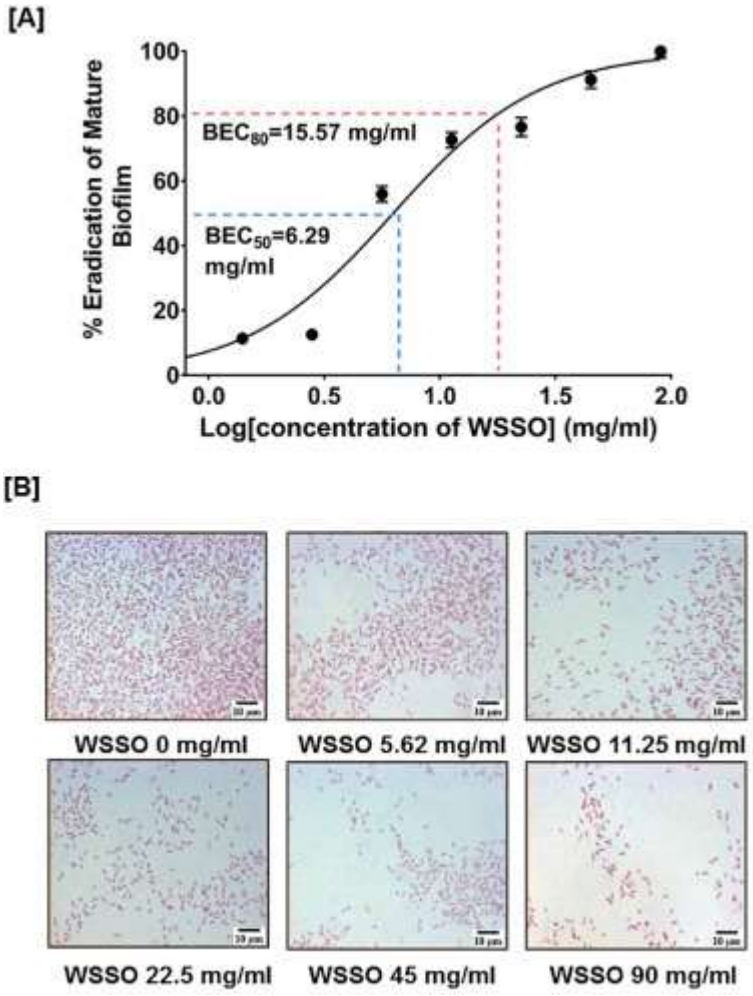

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Figure 7. WSSO efficiently disrupts mature biofilm

[A] Normalized dose-response curve showing the efficiency of WSSO in disrupting mature biofilm. Dose required for 50 % disruption of mature biofilm was calculated through linear regression and marked accordingly in the graph. [B] Representative microscopic images of Gram stained WSSO treated and untreated mature biofilms.

Discussion

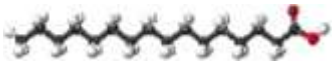
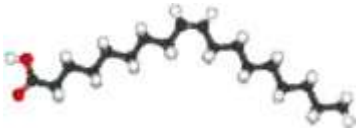
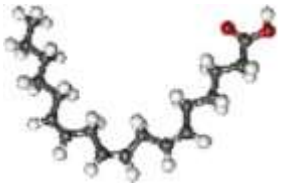
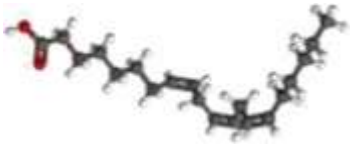
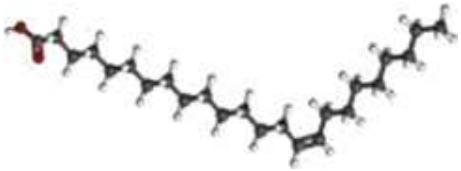
The alarming rate at which drug resistance is rising in Salmonella spp necessitates identification of alternatives to the current antibiotics against this bacterium, particularly, when

current therapies seem to be inadequate in managing Salmonella infection (Britto et al., 2018). Through this study, we have observed that WSSO could not only prevent growth but also efficiently inhibit initiation of biofilm formation and disrupt mature biofilms of S. enterica. However, its ability to prevent biofilm maturation was rather less pronounced. Delving into the possible underlying mechanisms behind these WSSO activities, we discovered that this oil significantly increased the bacterial membrane permeability. In fact, a double dose of the IC_{50} was equally efficient as the antibiotic chloramphenicol in increasing the membrane permeability. Since, FAs are already known to mimic different DSFs responsible for biofilm formation through affecting the motility of the bacteria, therefore, we checked the effect of WSSO on the communal locomotion of S. enterica through swimming and swarming assays. We noticed significant reduction, achieving almost no bacterial motility.

The observed IC_{50} of WSSO against S. enterica (5.78 mg/ml) falls in the range of minimum inhibitory concentrations reported for different plant extracts and natural compounds (Mostafa et al., 2011). WSSO was observed to be toxic against different mammalian cell lines, like, THP-1, A431, and RAW264.7 only at concentrations above 27 mg/ml, thereby, indicating suitability of its usage in humans and livestock, the main targets of Salmonella infections (Balkrishna et al., 2020). WSSO treatment increases membrane permeability clearly suggesting that the bacterial membrane is compromised. FAs are known to interact with bacterial cell membrane to reduce its integrity. Most of the natural compounds from herbal extracts disrupt the bacterial membranes (Lou et al., 2011).

WSSO is a mixture of long chain both saturated and unsaturated FAs with carbon chains ranging from C-16 to C-24. It is enriched in linoleic (54%), oleic (23%), palmitic (13%), and stearic (4%) acids as evident from gas chromatography-flame ionized detection (GC-FID) analysis. Ecosatrienoic and nervonic acids were also present, but in very small quantities (Figure 1; Table 2) (Balkrishna et al., 2020). While palmitic and stearic acids are saturated FAs, the others are unsaturated ones. Linoleic, stearic and oleic acids are already reported for their bactericidal activity against different forms of H. pylori and is found to be associated with H. pylori cell membrane (Jung et al., 2015). Palmitic, oleic and stearic acids were individually potent against Salmonella spp. (Zhang et al., 2016). Our observations also suggest that WSSO disrupts bacterial membrane with resultant leakage of K^+ and nucleotides. Overall these reports support our observations that FAs destabilize bacterial cell membranes which, in turn, leads to the leakage of intracellular components (Yoon et al., 2018). FAs as alternatives to antibiotics are attractive because of their non-specific targets, unlike antibiotics. Non-specific targets minimize the risk of developing resistant bacterial strains (Desbois and Smith, 2010). The observed antibacterial effect of WSSO is attributed to the FAs present in it. Single chain FAs, like the ones present in WSSO, when get incorporated into the cell membrane, decrease interactions between intra-membrane phospholipids. This, in turn, results in increased membrane fluidity and permeability. Besides, linoleic acid is known to inhibit enzymes involved in FA synthesis, thereby, exerting a negative effect on the membrane formation (Yoon et al., 2018).

Table 2. Fatty acids present in WSSO detected through gas chromatography-flame ionized detector (GC-FID)

WSSO Component	Molecular Formula	Chemical Structure	Content (% w/w)
Palmitic acid (Hexadecanoic acid)	$C_{16}H_{32}O_2$		12.64
Stearic acid (Octadecanoic acid)	$C_{18}H_{36}O_2$		4.07
Oleic acid (Cis-9-Octadecanoic acid)	$C_{18}H_{34}O_2$		23.16
Linoleic acid	$C_{18}H_{32}O_2$		54.15
11,14,17-Ecosatrienoic acid	$C_{20}H_{34}O_2$		0.66
Nervonic acid	$C_{24}H_{46}O_2$		0.38

Note: These data were obtained and published in our previous research paper Balkrishna et al. (2020) (Balkrishna et al., 2020).

Biofilm formation in Salmonella spp. significantly enhances its drug resistance (González et al., 2018). We evaluated the biofilm forming capacity in S. enterica strain and observed a moderate to strong biofilm forming propensity that matched earlier reports (Beshiru et al., 2018; Trmcic et al., 2018). Biofilm formation facilitates effective colonization of the luminal wall of the intestine leading to enhanced bacterial virulence, at the same time, imparting tolerance in the bacteria against hostile enteric environment. These chain of events are known to perpetuate into severe gastro-enteric infections in humans and have been recognized, over the past decade, as factors behind failure in infection management (MacKenzie et al., 2017). Transition to a sessile biofilm dominated lifestyle was associated with increase in the levels of saturated fatty acids in the cell membrane of Salmonella spp. (Dubois-Brissonnet et al., 2016). It is quite possible that in the presence of WSSO, that contains mainly unsaturated FAs, the bacterial membrane

populated with these single chain unsaturated FAs that eventually increase its permeability as already mentioned above (Yoon et al., 2018). The IC_{50} and BIC_{50} of WSSO were comparable, indicating equivalent antibacterial and antibiofilm efficacies. WSSO effectively inhibited motility in S. enterica. Biofilm formation depends on bacterial motility and efficient quorum sensing communication system within the bacterial colony. Unsaturated FAs are reported to jeopardize both of these in Acinetobacter baumanni, another Gram-negative pathogen popular for its nosocomial acquisition (Nicol et al., 2018). A hexane extract from ground beef containing a blend of palmitic, stearic, oleic and linoleic acids inhibited biofilm formation in Vibrio harveyi and Escherichia coli. Similarly, palmitic and oleic acids are known to affect the expressions of motility and quorum sensing related genes (Kumar et al., 2020). Based on these reports together with our observations, we speculate that a similar method is likely, to be employed by WSSO in inhibiting biofilm formation in S. enterica. It is worth to note that FAs act as signals for dispersion of mature biofilms, under normal conditions, to seed the formation of new biofilms (Davies and Marques, 2009). This is yet another caveat in the bacterial biofilm formation that is very effectively targeted by FAs to destroy mature biofilms when they are used at therapeutic doses (Marques et al., 2015). Putting all our observations in perspective, we proposed a hypothetical model describing the possible modes of action of WSSO as an anti-bacterial and anti-biofilm agent (Figure 8)

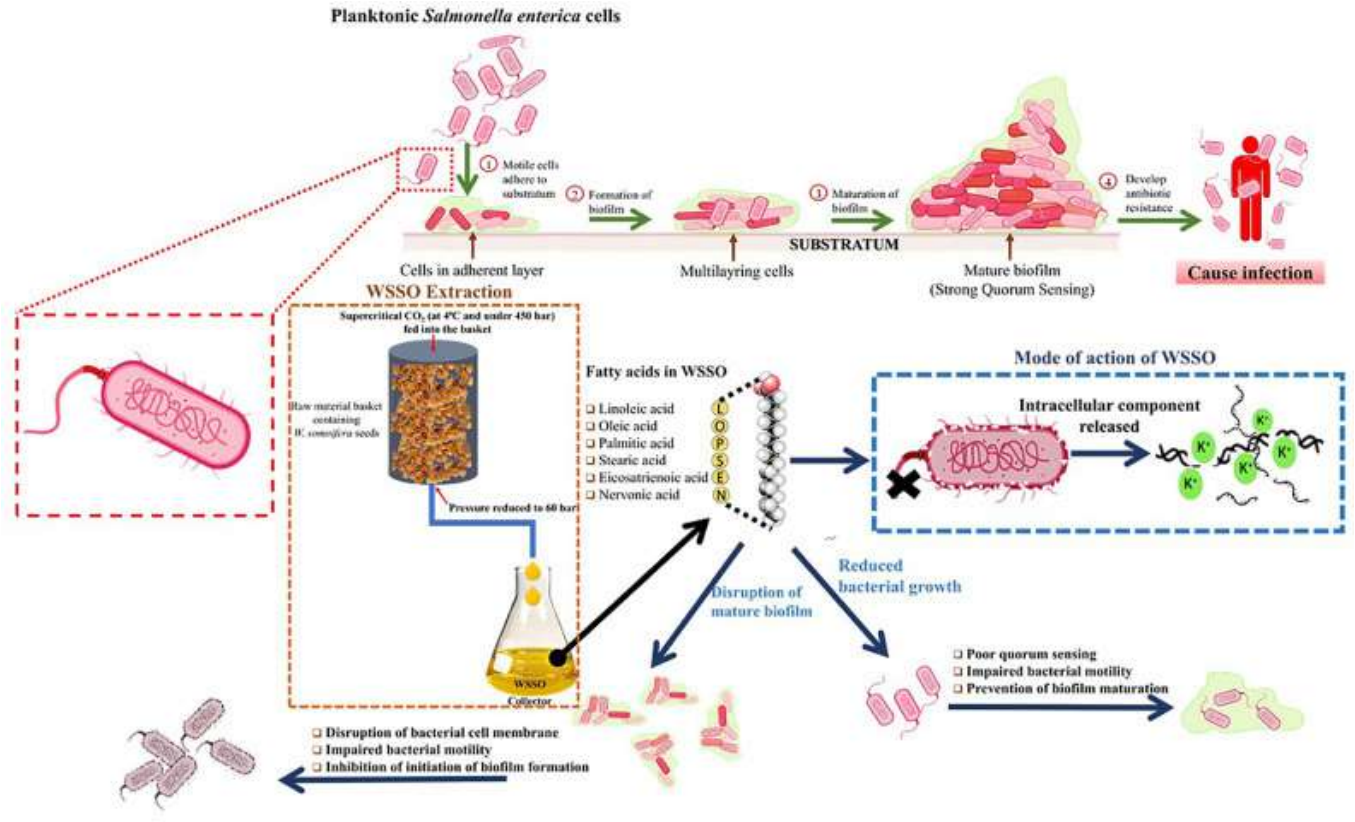


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Figure 8. WSSO efficiently disrupts mature biofilm

Schematic of a hypothetical model, as proposed based on the observations of this study, depicting a plausible mode of action of WSSO as an antibacterial and antibiofilm agent against Gram-negative pathogen, S. enterica.

Bacterial outer membrane is an evolving antibacterial barrier in Gram-negative bacteria which plays a significant role in imparting resistance against several antibiotics which are otherwise effective against Gram-positive bacteria (MacNair and Brown, 2020). Outer membrane disruptors are being increasingly recognized as an important component of combinatorial treatment, which is based on the principle of membrane perturbation followed by antibiotic treatment (Ma et al., 2019; MacNair and Brown, 2020; Vaara, 1992). Our observations implicate that WSSO is capable of perturbing the outer membrane of S. enterica. The outer leaflet of the membrane of enteric bacteria, including that of S. enterica, lacks glycerophospholipids, thereby preventing diffusion of hydrophobic solutes across it. This leaflet is a highly ordered quasi-crystalline structure composed of only lipopolysaccharide (LPS) (Vaara, 1992). Such lipid asymmetry of the outer membrane imparts its barrier function, fortifying it against antibiotics and bile salts (May and Grabowicz, 2018). The LPS sequestered in the outer leaflet of the outer membrane facilitates bridging via their negatively charged saccharide portions through divalent cations. Colistin, from polymyxin group of antibiotics, that disrupts these intermolecular bridging reactions, is used as an outer membrane disruptor. Unfortunately, colistin is not spared from antibiotic resistance due to evolved LPS with less negative charges on their saccharide portions (May and Grabowicz, 2018). Therefore, lipophilic substances that can disrupt LPS-mediated fortification, without specifically targeting any step of out membrane biosynthesis, is likely to survive the rapidly evolving resistance. While, we are short of insight into the antibacterial effect of WSSO, nevertheless, intuitive thinking points towards a possibility that this oil, being rich in long chain FAs (saturated and unsaturated, alike) is presumably allowed to diffuse into the outer membrane and consequently, disrupt its compactness.

Conclusion

There have been several reports on antibacterial FAs. But, a mix of such antibacterial FAs obtained from an herbal source is probably reported for the first time in this study. The effective dose of WSSO is significantly lower than the one that showed inhibition of mammalian cells. This implies that WSSO would be a safe alternative to current antibioticsfor systematic development into a therapeutic against drug-resistant bacteria. In conclusion, this study offers the preliminary evidences in favor for developing WSSO it into a clinically usable antibacterial agent.

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CRediT authorship contribution statement

Acharya Balkrishna: Conceptualization, Funding acquisition, Resources. **Ashish Kumar Gupta:** Investigation, Methodology, Validation, Visualization, Writing – original draft. **Kanchan Singh:** Investigation, Methodology, Validation, Visualization. **Swati Haldar:** Supervision, Investigation, Visualization, Writing – review & editing. **Anurag Varshney:** Conceptualization, Supervision, Project administration, Writing – review & editing.

Declaration of Competing Interest

The author(s) declare no competing interests and that this research was conducted with no commercial or financial relationships that can be construed as a potential conflict of interest.

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